ATTORNEY DOCKET NO. 23016.0002US
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	
Wynick, David	)	Art Unit: 1647
	)	
Application No. 09/230,463	)	Examiner: Gucker, S.
	)	
Filing Date: January 22, 1999	)	Confirmation No. 4323
	)	
For: "GALANIN"	)	

DECLARATION UNDER 37 C.F.R. § 1.132

1. I am Professor in the Department of Neurosciences at Case Western Reserve University, Ohio, USA.

2. I have been associated with teaching and research in the subject of Neurosciences for almost thirty years and have published approximately 100 peer-reviewed papers and 25 review articles and chapters during this time. Examples of these publications together with details of my education are given in the short version of my *curriculum vitae* which is attached and shown as Exhibit A.

3. My research relates to neurochemical plasticity in adult neurons. In recent years, my laboratory has focused on the molecules and cells involved in altering neuronal gene expression in response to axonal injury. The galanin peptide has been one molecule of long-standing interest and a major focus of my laboratory.

4. I am familiar with the work of David Wynick in the field of galanin and nerve regeneration.

5. I have reviewed the specification of US Patent Application Serial No. 09/230,463 ("the patent application"). I understand the technology described in the patent application. In

particular, I have reviewed claim 18 of the patent application and its meaning is clear to me.

6. I have also reviewed the publication "The effects of pretreatment with tachykinin antagonists and galanin on the development of spinal cord hyperexcitability following sciatic nerve section in the rat" by Luo, L. and Wiesenfield-Hallin, Z. (1995) Neuropeptides **28** 161-166 ("the Luo publication"). I understand the experiments described in that publication and the implications of the data resulting from those experiments.

7. In addition, I have reviewed the Office Action mailed on 10th March 2005, particularly item 5 of the Action in which the Examiner rejected claims 18 and 25-26 under 35 U.S.C. 103(a) as being unpatentable over the Luo publication in view of Zhang *et al.* (J. Neurocytology (1993) **22** 342-381).

8. The Luo publication demonstrates that galanin inhibits spinal cord electrical hyper-excitability for 60 minutes following nerve section (see Figure 3), at which point the animals were sacrificed. Galanin was administered 30 minutes before the nerve injury as a one-off bolus injection of 2.4nM (low dose) directly into the space surrounding the bottom part of the spinal cord (intrathecal (IT) administration into the lumbar enlargement, see page 163, paragraph headed "Effect of galanin" and Figure 3 of the Luo publication). The Luo publication deals exclusively with neuropathic pain and spinal cord excitability, not with peripheral nerve regeneration as claimed in the patent application. The mechanism by which the galanin rapidly alters pain activity is most likely by direct modulation of spinal cord neuronal firing rather than at the level of the dorsal root ganglion (DRG).

9. When damage or injury to sensory or motor nerves (in this case the sciatic nerve) occurs, this triggers a cascade of molecular events within the cell bodies of that nerve, in this case the DRG, which in turn attempts to repair the damage and restore the normal function of the nerve, so-called nerve regeneration. The Protein Kinase C (PKC) and MAP Kinase (MAPK) intra-cellular signalling cascades have both been shown to up-

regulate after nerve injury and are vital for nerve regeneration (Klinz & Heumann (1995) J. Neurochem. **65** 1046-53; Kiryu et al. (1995) Brain Res. Mol. Brain Res. **29** 147-56). At the 1996 priority date of the patent application, no published literature existed to indicate that galanin speeded up nerve regeneration, nor that it activated PKC or MAPK.

10. For regeneration to have occurred in the animals used in the experiments documented in the Luo publication during the 90 minute period before the animals were sacrificed, the galanin would have had to gain access to the cell bodies in the DRG, since this is where the intra-cellular pro-regenerative machinery resides (Terenghi (1994) J. Anatomy (Pt I) 1-14). The only way that galanin, when applied to the space surrounding the bottom part of the spinal cord, could have reached the cell bodies of the sensory neurons in the DRG which is where "...the preliminary beginnings of regeneration..." would have occurred, is by direct uptake of the galanin by the nerve terminals in the dorsal horn of the spinal cord. The cell bodies of the sensory neurons of the DRG lie outside the central nervous system (CNS), whilst the spinal cord is part of the CNS. The cerebrospinal fluid (CSF) that bathes the spinal cord is not in contact with the DRG and thus galanin could not have reached the DRG by passive diffusion.

11. There are well documented and active transport mechanisms in sensory neurons that move proteins from the nerve terminals of the spinal cord or the ends of the peripheral axons of the sciatic nerve to the cell bodies in the DRG, termed retrograde transport. A number of these retrograde transport mechanisms for Nerve Growth Factor (NGF) and Horseradish Peroxidase (HRP) have been extensively studied and characterized. There is good agreement between these published papers that the rate at which these retrograde transport processes move proteins from the rat dorsal horn of the spinal cord to the cell body in the DRG is a maximum rate of 7.5 mm/hour (range 2.5 – 7.5 mm/hr, Yip and Johnson, Jr. (1986) J. Neurocytol. **15** 789-98; Richardson and Riopelle. (1984) J. Neurosci. **4** 1683-9).

12. Michael et al. (J. Neurosci. (1997) **17** 8476-8490) found the nerve root of adult male Wistar rats (200-400g body weight) to be 17 mm in length. Similarly, Baba et al. (Baba et al. (1999) J. Neurosci. **2** 859-867) found the dorsal root to be between 18-20 mm in

length in adult male Sprague-Dawley rats weighing 300-350g. Based on this, assuming the maximum rate of retrograde transport of galanin from the dorsal horn of the spinal cord to the DRG is 7.5 mm/hr and the length of the nerve root between the dorsal horn of the spinal cord and the DRG is at least 17mm in length, then the galanin would only have been transported 11.25 mm in the 90 minutes after galanin was administered before the animals were sacrificed (i.e. about two thirds of the way to the DRG). Galanin could not, therefore, have even begun to affect regeneration in the DRG cell bodies by the time the experiment was terminated.

13. In addition, the concentration of galanin would have immediately and rapidly begun to fall straight after the administration of the bolus injection of 2.4nM galanin into the lumbar enlargement of the spinal cord. This would have occurred within seconds, as the galanin would immediately be diluted by the CSF and almost immediately would also have started to be degraded by proteolytic enzymes in the CSF (Bedecs et al. (1995) Neuropeptides **29** 137-43). Therefore, even in the highly unlikely event that a small proportion of the galanin that was administered by bolus-dose to the spinal cord did reach the DRG by retrograde transport, the final dose would be substantially lower (in the sub-nanomolar range) than the originally administered IT (page 163 and Figure 3 of the Luo publication). In contrast, the dose of galanin demonstrated to stimulate nerve outgrowth from sensory neurons by direct application in cell culture to the DRG cell body is 100nM galanin (Mahoney et al. (2003) J. Neurosci. **23** 416-421). In light of this, the effective concentration of galanin that would have reached the DRG would have been at least 100-fold lower than that necessary to stimulate regeneration, again making it highly unlikely that galanin could have even begun to affect regeneration in the DRG cell bodies by the time the experiment was terminated.

14. For these reasons, it is my view that one of ordinary skill in the art would have no incentive, on reading the Luo publication, to imagine that galanin-induced nerve regeneration of the severed sciatic nerve would have begun during the time period utilized in the experiments of Luo et al. In addition, in light of the Luo et al. disclosure and on reading the disclosure in Zhang et al. that galanin may be suitable for use as an

analgesic in humans, one of ordinary skill in the art would have no motivation to administer a galanin agonist in a method for the treatment of peripheral nerve damage, wherein the peripheral nerve damage is treated by nerve regeneration, as claimed in claim 18 as amended with the most recently filed Applicant's response.

15. I further declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 6/1/05

Professor Richard E. Zigmond

EXHIBIT A

BIOGRAPHICAL SKETCH	
NAME RICHARD E. ZIGMOND	POSITION TITLE PROFESSOR OF NEUROSCIENCES

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Harvard College, Cambridge MA	BA cum laude	1962-66	Biology
Rockefeller U., NY, grad. stud.with Bruce McEwen	Ph.D.	1966-71	Neuroscience
Rockefeller U., NY, postdoc.with Don Pfaff		1971-72	Neuroendocrinology
U. of Cambridge, UK, postdoc. with Leslie Iversen		1972-75	Neurochemistry
Harvard Medical School/Children's Hospital, (Sabbatical with Michael Greenberg)		2000-01	Molecular Biology

Positions and Honors

Appointments: Assist. Prof. (1975-81), Assoc. Prof. (1981-89) of Pharmacology, Harvard Medical School; Instructor in Neurobiology of Behavior at Cold Spring Harbor Lab. (1979-82); Prof. of Neurosci., Case Western Reserve Univ. (CWRU) School of Medicine (1989-present); Instructor in Neurobiology at Marine Biology Lab. (1981-84); Program Director, NIH Postdoctoral Training Program in Devel. Neurol. (1981-89; Harvard Med. Sch.); Instructor on Review and Update in Neurobiology for Neurosurgeons (1984, 86, 88); Chair, Committee on Appointments and Tenure, Department of Neurosciences, CWRU (1991-present); Chair, Gordon Conference on Neural Plasticity (1991); Program Committee, Society for Neuroscience (1991-93); Acting Chair, Department of Neurosciences, 1992-93).

Fellowships and Special Grant Awards: Pop. Council Fellowship in Mammalian Reproduction (1971-72); British-American Heart Found Fellowship (1972-73); Sloan Found. Fellowship in Neurochem. (1972-74); Klingenstein Fellowship in the Neurosciences (1987-1990); Mellon Found. Faculty Award (1976-1977); NIMH Research Scientist Development Award (1977-87); Javits Neuroscience Investigator Award (1987-94); NIMH Research Scientist Award (1987-94).

Grant Review Committees: External review committee for the Lab. of Developmental Neurobiology NICHD (1985, 1990); Study Section for Tobacco-Related Disease Research Program of the Univ. of California (1993, 1994); Ad hoc Reviewer, Neurology C Study Section (Neuro C; 1995, 1996); Member, Neurological Sciences 1 Study Section (NLS1) and Molecular Developmental and Cellular Neurosciences Study Section (MDCN7; 1996-2000); Reviewer of Research at the Burke Medical Research Institute (1998).

EXHIBIT A

Editorial Boards: *J. Neurosci. Meth.* (1978-1998), *J. Neurosci.* (1985-91), *Ann. Rev. Neurosci.* (1986-90), *TINS* (1986-90), *New Biol.* (1989-91), *Adv. in Neurosci.* (1989-present), *Neuroscience* (1996-present), *J. Mol. Neurosci.* (1997-present), *Autonomic Neuroscience: Basic and Clinical (*formerly *J. Auton. Nerv. Syst.* (1998-), *NeuroSignals* (formerly *Biological Signals and Receptors* (2001-present).

Selected peer-reviewed publications since 1998.

1. Rao MS, Sun Y, Escary JL, Perreau J, Patterson PH, Zigmond RE, Brulet P, Landis SC. Leukemia inhibitory factor mediates an injury response but not a developmental transmitter switch in sympathetic neurons. Neuron 11:1175-1185, 1993.
2. Sun Y, Rao MS, Zigmond RE, Landis SC. Regulation of vasoactive intestinal peptide expression in sympathetic neurons in culture and after axotomy: The role of cholinergic differentiation factor/leukemia inhibitory factor. J Neurobiol 25:415-430, 1994.
3. RC, Shadiack AM, Bennett TA, Sedwick CE, Zigmond RE. Changes in the macrophage population of the rat superior cervical ganglion after postganglionic nerve injury. J Neurobiol 27:141-153, 1994.
4. Sun Y, Landis S, Zigmond R. Signals triggering the induction of leukemia inhibitory factor in sympathetic superior cervical ganglia and their nerve trunks after axonal injury. Mol Cell Neurosci 7:152-163, 1996.
5. Hyatt-Sachs H, Bachoo M, Schreiber R, Vaccariello SA, Zigmond RE. Chemical sympathectomy and postganglionic nerve transection similarly increase galanin and VIP mRNA but not the peptides themselves. J Neurobiol 30:543-555, 1996.
6. Sun Y, Zigmond RE. Leukemia inhibitory factor induced in the sciatic nerve after axotomy is involved in the induction of galanin in sensory neurons. Europ J Neurosci 8:2213-2220, 1996.
7. Zigmond, RE. Retrograde and paracrine influences on neuropeptide expression in sympathetic neurons after axonal injury. In: Cytokines and the CNS: Development, Defenses and Disease (RM Ransohoff, EN Benveniste, Eds) CRC Press, Boca Raton, pp. 169-186, 1996.
8. Zigmond RE, Hyatt-Sachs H, Mohney RP, Schreiber RC, Shadiack AM, Sun Y, Vaccariello SA. Changes in neuropeptide phenotype after axotomy of adult peripheral neurons and the role of leukemia inhibitory factor. Perspec Dev Neurobiol 4:75-90, 1996.
9. Zhou Y, Deneris E, Zigmond RE. Differential regulation of levels of nicotinic receptor subunit transcripts in adult sympathetic neurons after axotomy. J Neurobiol 34:164-178, 1998.
10. Shadiack AM, Zigmond RE. Galanin induced in sympathetic neurons after axotomy is anterogradely transported toward regenerating nerve endings. Neuropeptides 32:257-264, 1998.
11. Shadiack AM, Vaccariello SA, Sun Y, Zigmond RE. Nerve growth factor inhibits sympathetic neuron's response to an injury cytokine. Proc Natl Acad Sci USA 95:7727-7730, 1998.
12. Mohney RP, Zigmond RE. Vasoactive intestinal peptide enhances its own expression in sympathetic neurons after injury. J Neurosci 18:5285-5293, 1998.
13. Zigmond RE, Mohney RP, Schreiber RC, Shadiack, AM, Sun Y, Vaccarriello SA, Zhou Y. Plasticity in gene expression in adult sympathetic neurons after axonal damage. Adv in Pharmacol 42:899-903, 1998.
14. Nagamoto-Combs K, Vaccariello S, Zigmond RE. The levels of LIF mRNA in a Schwann cell line are regulated by multiple second messenger pathways. J Neurochem 72:1871-1881, 1999.
15. Mohney RP, Zigmond RE. Galanin expression is decreased by cAMP-elevating agents in cultured sympathetic ganglia. NeuroReport 10:1221-1224, 1999.

EXHIBIT A

16. Rittenhouse A R., Zigmond RE. The role of N- and L-type calcium channels in the depolarization-induced activation of tyrosine hydroxylase and release of norepinephrine by sympathetic cell bodies and nerve terminals. J Neurobiol 40:137-148, 1999.
17. Boeshore KL, Luckey CN, Zigmond RE, Large TH. TrkB isoforms with distinct neurotrophin specificities are expressed in predominantly non-overlapping populations of avian dorsal root ganglion neurons. J Neurosci 19:4739-4747, 1999.
18. Ip NY, Zigmond RE. Synergistic effects of muscarinic agonists and secretin or vasoactive intestinal peptides on the regulation of tyrosine hydroxylase activity in sympathetic neurons. J Neurobiol 42:14-21, 2000.
19. Bibevski S, Zhou Y, McIntosh J, Zigmond R, Dunlap M. Functional nicotinic acetylchoine receptors that mediate ganglionic transmission in cardiac parasympathetic neurons. J. Neurosci 20:5076-5082, 2000.
20. Zigmond RE. Neuropeptide action in sympathetic ganglia: Evidence for distinct functions in intact and axotomized ganglia. Ann N Y Acad Sci.921:103-108, 2000.
21. Shadiack A, Sun Y, Zigmond R. Nerve growth factor antiserum induces axotomy-like changes in neuropeptide expression in intact sympathetic and sensory neurons. J. Neurosci. 21:363-371, 2001.
22. Zhou Y, Deneris E, Zigmond R. Nicotinic acetylcholine receptor subunit proteins alpha7 and beta4 decrease in the superior cervical ganglion after axotomy. J Neurobiol. 46: 178-192, 2001.
23. Schreiber RC, Krivacic K, Kirby, B, Vaccariello SA, Tani M, Ransohoff RM, and Zigmond RE. Monocyte chemoattractant peptide- 1(MCP-1) is rapidly expressed by sympathetic ganglion neurons following axonal injury. NeuroReport 12:601-606, 2001.
24. Zigmond RE. Can galanin also be considered as growth-associated protein 3.2? TINS 24:494-496, 2001.
25. Takasu, AM. Dalva MB, Zigmond R, Greenberg, ME. Science. EphB receptors modulate NMDA receptor-dependent calcium influx and gene expression. Science 295: 491-495, 2002.
26. Schreiber RC, Boeshore K, Vaccariello SA, Shadiack, Zigmond RE. A comparison of the changes in the non-neuronal cell populations of the superior cervical ganglia following decentralization and axotomy. J. Neurobiol. 53: 68-79, 2002.
27. Zigmond R.E. Plasticity in the autonomic nervous system: Responses of adult sympathetic neurons to injury. In: Handbook of Autonomic Nervous System in Health and Disease (J Licinio and L Bolis, Eds) Marcel Dekker, New York, pp. 167-184, 2002.

Research Support

Sponsor: National Institutes of Health **Award Number:** NS12651
Dates: 7/1/2003-6/30/2004 **Amounts:** Current Direct Costs: 0
Title: Experience and the Neurochemistry of the Synapse
Percent Effort: 35%
Brief description of the project: To identity the transmitter responsible for the non-cholinergic activation of TH in the SCG after preganglionic nerve stimulation, determine whether such nerve stimulation alters neuropeptide expression, determine whether PACAP and VIP are involved in feedback mechanisms regulating their own expression, examine the signals triggering the changes in nAChR receptor subunit mRNA expression in axotomized SCG neurons, determine if changes occur at the receptor level, and ask whether a phenomenon comparable to "disuse supersensitivity" is seen in these receptors as a result of changes in afferent nerve stimulation.

EXHIBIT A

Sponsor: National Institutes of Health **Award Numbers:** NS17512
Dates: 5/15/03-4/30/07 **Amounts:** Current Direct Costs: $237,500
Title: Recovery of Function after Neural Damage
Percent Effort: 35%

Brief description of the project: To determine the cellular and molecular changes that occur in peripheral neurons in the context of regeneration. We have been notified by the NINDS that this application will be refunded.

J. Anat. (1999) 194, pp. 1–14 Printed in the United Kingdom

Review

Peripheral nerve regeneration and neurotrophic factors

GIORGIO TERENGHI

Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead, Sussex, UK

(*Accepted 27 August 1998*)

ABSTRACT

The role of neurotrophic factors in the maintenance and survival of peripheral neuronal cells has been the subject of numerous studies. Administration of exogenous neurotrophic factors after nerve injury has been shown to mimic the effect of target organ-derived trophic factors on neuronal cells. After axotomy and during peripheral nerve regeneration, the neurotrophins NGF, NT-3 and BDNF show a well defined and selective beneficial effect on the survival and phenotypic expression of primary sensory neurons in dorsal root ganglia and of motoneurons in spinal cord. Other neurotrophic factors such as CNTF, GDNF and LIF also exert a variety of actions on neuronal cells, which appear to overlap and complement those of the neurotrophins. In addition, there is an indirect contribution of GGF to nerve regeneration. GGF is produced by neurons and stimulates proliferation of Schwann cells, underlining the close interaction between neuronal and glial cells during peripheral nerve regeneration. Different possibilities have been investigated for the delivery of growth factors to the injured neurons, in search of a suitable system for clinical applications. The studies reviewed in this article show the therapeutic potential of neurotrophic factors for the treatment of peripheral nerve injury and for neuropathies.

Key words: Nerve injury; peripheral neuropathy.

PERIPHERAL NERVE INJURY AND REGENERATION

The process of nerve injury and regeneration involves many interactions between cellular elements and between these and the extracellular matrix. Immediately after axotomy, the proximal and distal nerve stumps retract, axoplasm leaks out and damaged membranes collapse. Macrophages are recruited to the site of the lesion during the first week postinjury (Perry et al. 1987), contributing to lysis and phagocytosis of myelin and subsequent Schwann cell proliferation (Beuche & Friede, 1984). Both cell types secrete mitogens and growth factors which play a role in axonal regeneration and remyelination (De Vries, 1993; Reynolds & Woolf, 1993).

In the distal axonal stump, Wallerian degeneration takes place during the first few days postinjury. The axons degenerate, their myelin sheath detaches and degrades, and the degradation products, together with macrophage secretion, stimulate the Schwann cells within the distal stump to proliferate within their basal lamina tubes (Salzer & Bunge, 1980; Salzer et al. 1980; De Vries, 1993), forming the bands of Büngner (Bunge, 1980). This proliferation continues for approximately 2 wk, with the Schwann cells forming a conduit which guides the regenerating axons to their target (Son & Thompson, 1995). Schwann cells are vital for the process of axonal regeneration (Hall, 1986, 1997), and one of their roles is as a source of neurotrophic factors (Heumann et al. 1987; Acheson et al. 1991; Sendtner et al. 1992). These diffuse from the distal stump across the injury area to exert a trophic influence on the axons regenerating from the proximal stump (Reynolds & Woolf, 1993). The contact with regenerating axons stimulates a second phase of Schwann cell proliferation, which is mediated by a neuronally derived trophic factor specific for glial

Correspondence to Dr G. Terenghi, Blond McIndoe Laboratories, Department of Surgery, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2QG, UK. Tel.: +44 0171 794 0500, ext. 3944; e-mail: terenghi@mcindoe.ucl.demon.co.uk

cells. However, if axonal regeneration is delayed, Schwann cells decrease progressively in number and become less responsive to axonal regeneration (Li et al. 1997; Terenghi et al. 1998).

In the proximal stump, the axons degenerate retrogradely as far as to the first node of Ranvier, creating a small area of wallerian degeneration. Within a few hours, the injured axons give rise to several neuronal sprouts (Wong & Mattox, 1991), whose number is in excess of the number of axons originally in the nerve fascicles. It has been proposed that this phenomenon is to maximise the chances for each neuronal cell reaching its target organ. Some of these sprouts will 'die back' through axonal pruning (Brushart, 1993) because of insufficient survival signal from the target organ, most probably in the form of growth factors. The terminal tip of the regrowing axons, or growth cone, responds to contact guidance cues and actively searches for a suitable matrix and environment to support the axonal growth (Bixby et al. 1988; Rutishauser, 1993). Schwann cells from the distal stump are the most effective substrate to produce and direct regeneration (Bixby et al. 1988), and successful axonal regeneration depends on the interaction between axons and glial cells (Hall, 1986, 1997). Although the release and presence of growth factors play an important part in the regeneration process, an interaction between Schwann cell membrane and growth cone is mediated by cell adhesion molecules (CAMS) expressed by both elements (Rutishauser, 1993). There is also evidence to suggest that nerve growth factor (NGF), and other growth factors, released by Schwann cells may potentiate axonal regeneration by upregulating neuronal CAMS expression (Friedlander et al. 1986; Seilheimer & Schachner, 1987).

During regeneration, the axons also respond selectively to tropic cues (Gu et al. 1995) and grow back preferentially towards the target organ which they originally innervated, although the mechanism regulating this specificity is not fully understood. Motor fibres will seek out and selectively innervate a motor distal axonal stump (Brushart et al. 1987; Brushart, 1988; Rath et al. 1991*a*). This phenomenon is thought to depend on the preferential expression of the L2 epitope by the myelinating Schwann cells associated with motor axons (Martini et al. 1994). The differential expression of this epitope on Schwann cells associated with sensory axons could provide a tropic cue for the motor axons to regenerate in the appropriate pathway. However, muscle sensory afferents retain a high degree of accuracy for reinnervation to the appropriate muscle (Madison et al. 1996) and sensory fibres reinnervate end organs of the same subtype originally innervated (Dykes, 1984). This is consistent with clinical finding that different types of sensation (i.e. pressure, pain, temperature, etc.) are not confused after reinnervation (Dellon, 1981; Terzis & Smith 1990). It would seem that sensory nerves lack topographic specificity (Rath et al. 1991*b*), but they display target organ specificity, derived from the presence of specific neurotrophic factors.

NEUROTROPHIC FACTORS

Cytokines and growth factors are a varied group of polypeptides produced by different cell types and showing overlapping actions (Hopkins & Rothwell, 1995; Rothwell & Hopkins, 1995). Although the 2 terms are often used as synonyms, the major distinction between the 2 is that cytokines are inducible while growth factor are constitutive. However, both are upregulated and particularly active in high-metabolism situations, such as trauma and inflammation. Cytokines and growth factors have been subdivided into families according to their sequence similarity and to the type of binding receptors (Hokpkins & Rothwell, 1995). Within the growth factor group, neurotrophic factors specifically influence neuronal activity by promoting development and maturation during embryonic life, and by sustaining maintenance during adult life and regeneration after injury. There are numerous neuronal growth factors, and it would be impossible to give here a comprehensive review. For this reason, this article will concentrate on the neurotrophins and on a few other growth factors, whose roles and actions have been shown to overlap and complement those of the neurotrophins in promoting peripheral neuron survival and regeneration.

The basic neurotrophic factor concept is defined by the hypothesis that trophic proteins are synthesised in the target tissues, and delivered to the neuronal soma via retrograde transport where they exert a trophic and survival effect (Purves, 1986; Oppenheim, 1991; Lindsay, 1996*a*). During fetal development, the supply of growth factors is limiting for neurons innervating various target tissues in order to select and sustain specific neuronal subpopulations, while the surplus neurons undergo cell death (Davies, 1996; Snider & Silos-Santiago, 1996). The first neuronal growth factor to be discovered, and the prototype for the concept of neurotrophic factors, is NGF, which is specific for a subset of primary sensory neurons and for sympathetic neurons (Levi-Montalcini, 1987).

NGF is produced by the target organs (Bandtlow et al. 1987; Barde, 1990, 1994; Anand et al. 1996), from where it is delivered to the neuronal cell bodies by retrograde axonal transport (Di Stefano et al. 1992) following binding to specific receptors and internalisation by the nerve terminals (Thoenen & Barde, 1980; Lewin & Barde, 1996). There is a wealth of information about the role and actions of NGF (see Rush et al. 1995 for review), which has also stimulated the research on other neurotrophic factors.

Other members of the neurotrophin family include brain derived neurotrophic factor (BDNF) (Barde et al. 1978), neurotrophin-3 (NT-3) (Ernfors et al. 1990) and neurotrophin 4/5 (NT-4/5) (Berkemeier et al. 1991; Hallbook et al. 1991). All these proteins have a high degree of amino acid homology with NGF (Glass & Yancopoulos, 1993; Maness et al. 1994). They share a low-affinity receptor p75 (Chao et al. 1986), to which they bind with equal affinity (Rodriguez-Tebar et al. 1990, 1992). Although the specific role of p75 is not completely understood, this receptor interacts with high affinity receptors belonging to the tyrosine kinase (trk) receptor family (Barbacid, 1994; Chao & Hempstead, 1995). Low and high affinity receptors are colocalised within the same neurons (Verge et al. 1992) and their interaction is crucial for the internalisation of neurotrophins within the neuronal terminals (Gargano et al. 1997). Three trk receptors have been identified, each specific for a different neurotrophin: trkA is the signal transducing receptor for NGF (Kaplan et al. 1991), trkB is specific for BDNF and NT-4/5 (Klein et al. 1989, 1992), while NT-3 binds preferentially to trkC (Lamballe et al. 1991). Cross-reactivity between NT-3 and other high affinity trk receptors has been reported, although the avidity of the binding and the potency of the neuronal response may be variable (Squinto et al. 1991; Davies et al. 1995; Rydén & Ibáñez, 1996).

All 3 trk receptors are distributed to discrete but partially overlapping subpopulations of primary sensory neurons, which give rise to different types of afferents and modulate specific sensory functions (McMahon et al. 1994; Wright & Snider, 1995; Bennett et al. 1996), while trkB and trk C are also present in spinal motoneurons (Ernfors et al. 1993; Funakoshi et al. 1993). Consistently, the target tissue innervated by the afferents of the different trk neurons express mRNA for the relevant neurotrophin (Davies et al. 1987; Maisonpierre et al. 1990; Ernfors et al. 1992; Buchman & Davies, 1993; Griesbeck et al. 1995). Hence the growth factors are retrogradely transported from the peripheral target organs to the appropriate neuronal cells (Di Stefano et al. 1992). Schwann cells in peripheral nerves also synthesise growth factors, and their synthesis is regulated by axonal contact or, in case of injury, loss of it (Reynolds & Woolf, 1993).

Approximately a third of primary sensory neurons do not express trk receptors, and can be identified by the binding of the lectin IB4. These neurons have been found to express Ret mRNA, the signal transduction component of the receptor for glial-derived neurotrophic factor (GDNF), a member of the transforming growth factor-β (TGF-β) superfamily (Lin et al. 1993). Ret is a common receptor for GDNF and neurturin, and it interacts with either of 2 other receptor subunits, GFRα-1 and GFRα-2 (Jing et al. 1996; Baloh et al. 1997). Ret receptors have been identified in small diameter primary sensory neurons, and colocalise with GFRα-1 and GFRα-2 subunits (Bennett et al. 1998). GDNF has a trophic effect on dorsal root ganglion cells as well as on motoneurons and autonomic neurons (Henderson et al. 1994; Buj-Bello et al. 1995; Matheson et al. 1997; Bennett et al. 1998).

Ciliary neurotrophic factor (CNTF) is a neuroactive cytokine which overlaps considerably with neurotrophin in terms of neuronal specificity, although its cellular localisation, receptor structure and signalling pathway are distinct from those of the neurotrophin family (Ip & Yancopoulos 1992; Richardson, 1994). CNTF is abundantly present in normal peripheral nerves, being localised mainly in the cytoplasm of myelinating Schwann cells (Friedman et al. 1992; Rende et al. 1992). The mechanism of release of CNTF from the Schwann cells is still unclear as the protein lacks the signal sequence generally associated with secreted proteins (Stockli et al. 1989). Its CNTFα receptor has been localised to muscle and spinal cord (Davis et al. 1991; Helgren et al. 1994) and consistently CNTF promotes survival of motoneurons in vitro and in neonatal animals following axotomy (Arakawa et al. 1990; Sendtner et al. 1990). Despite these survival-promoting effects, the role of CNTF in normal adult neuronal trophic support is still unresolved, and it has been speculated that CNTF may act as an 'injury factor', being released by glial cells in response to injury (Adler, 1993).

A newly discovered growth factor which has been linked to peripheral neuronal survival is leukaemia inhibitory factor (LIF) (Yamamori et al. 1989). In vitro, the actions of LIF on sympathetic neurons are very similar to those of CNTF (Yamamori et al. 1989; Nawa et al. 1990). Like other neurotrophic factors, LIF is retrogradely transported by a subpopulation of small diameter neurons in dorsal root ganglia (DRG).

A large proportion of these neurons are also positive for trkA and CGRP, while the remaining are labelled by IB4 (Thompson et al. 1997). This overlap between neurons accumulating LIF and those responsive to NGF is much wider that that described for cells showing receptors for neurotrophin and GDNF (Michael et al. 1997; Bennett et al. 1998). These findings and the demonstration that sympathetic neurons transport retrogradely both to NGF and NT-3 from peripheral target tissues (Zhou et al. 1997) are consistent with the intriguing possibility that there may be multiple growth factor control on neuronal functions and survival.

Glial growth factor (GGF) is a trophic factor specific for Schwann cells rather than neurons, but it has a significant role in the interaction of the 2 cell types. GGF was originally characterised as a Schwann cell mitogen, and only subsequently it was found to be part of a family of proteins known as neuregulins, encoded in a single gene and differentially spliced during transcription (Marchionni et al. 1993). The neuregulin proteins acts via heterodimers of erbB2, erbB3 and erbB4 receptors (Carraway & Burden, 1995) which are also present on glial cells. Neuregulin mRNA is expressed by primary sensory neurons, motoneurons and sympathetic neurons (Marchionni et al. 1993; Chen et al. 1994). During development, GGF is trophic for Schwann cell precursors and stimulates cell proliferation (Dong et al. 1995) and is critical for the survival of Schwann cells in developing neuromuscular junctions (Tranchtenberg & Thompson, 1996). In adults, GGF increases Schwann cell motility and proliferation, the 2 effects being dependent on the concentration of growth factor available to the glial cells (Mahanthappa et al. 1996). Hence GGF may promote neuronal survival and proliferation indirectly, by promoting glial cells-neuron interaction (Reynolds & Woolf, 1993).

It is clear that trophic factors play an important role during development as well as in adult life. It has been found that some neuronal populations may be sustained during development by a specific trophic factor, but they switch to a different trophic support in adult life as shown by the changing pattern of expression of different neurotrophins (Maisonpierre et al. 1990; Zhang et al. 1994; Oakley et al. 1995; Verdi et al. 1996; Belliveau et al. 1997; Rush et al. 1997). Similarly, the IB4-labelling subpopulation of primary sensory neurons start to express the Ret receptor for GDNF perinatally, after the down-regulation of NGF-binding trkA which is prevalent in these cells during fetal development. In adult life, IB4-labelled cells do not express trk receptors and are totally dependent on GDNF trophic support (Molliver et al. 1997). In adults, peripheral nerve injury seems to recapitulate, at least partially, the events which occur during development, with regulatory changes of trophic factors and their receptors.

NEUROTROPHIC FACTORS AND NEURONAL RESPONSE TO INJURY

Primary sensory neurons

In adults, peripheral nerve injury is followed by significant neuronal cell death in the DRG, with 20–40% of neurons within the DRG being lost (Ardvidsson et al. 1986; Schmalbruch, 1987; Himes & Tessler, 1989; Liss et al. 1994), most probably due to apoptosis (Edström et al. 1996; Groves et al. 1997). There is also downregulation of trk receptor expression in response to a peripheral nerve lesion and to availability of neurotrophic factors (Ernfors et al. 1993; Krekowski et al. 1996). These changes are counteracted by the exogenous administration of neurotrophins (Verge et al. 1996), which can also improve the physiological activity of the sensory neurons (Munson et al. 1997).

Following axonal injury, Schwann cells of the distal stump upregulate the synthesis of NGF, BDNF, NT-4 and p75, but not NT-3 (Heumann et al. 1987; Meyer et al. 1992; Funakoshi et al. 1993; Anand et al. 1997). The increase of the low-affinity receptors is detectable before axonal degeneration (Robertson et al. 1995) and it is thought that the combined NGF and p75 increase may promote the proliferation and migration of Schwann cells (Anton et al. 1994). However, the neurotrophin levels made available at the site of injury are not sufficient to compensate for the lack of target derived neurotrophic factor to maintain cell survival (Johnson et al. 1985; Heumann et al. 1987). In those neurons which survive the initial insult, the regenerative response is accompanied by a shift in synthesis of mRNA, which is best characterised by the modification in phenotypic neuropeptide expression in primary sensory neurons. There is a downregulation of calcitonin gene related peptide (CGRP) and substance P (SP), whose expression in small and medium size sensory neurons is regulated by NGF (Lindsay & Harmar, 1989). Changes of retrogradely transported signal not linked to NGF also cause upregulation of vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and galanin in well defined neuronal subpopulations (Kashiba et al. 1992; Hökfelt et al. 1994; Zhang et al. 1995; Wakisaka et al. 1996). The link between trophic factors and peptide phenotypic expression has been used as a marker of

neuronal injury and of the response of subpopulations of DRG neurons to trophic factors. Evidence from in vitro studies support the contention that neuropeptide alterations are regulated by neurotrophic factors (Lindsay & Harmar; 1989; Mulderry, 1994; Kerekes et al. 1997). Consistently, in vivo administration of NGF reverses the decrease of CGRP and SP expression in small diameter primary sensory neurons due to nerve injury (Fitzgerald et al. 1985; Wong & Oblinger, 1991; Verge et al. 1995), while GDNF acts on somatostatin expression in small size neurons which are not responsive to NGF (Bennett et al. 1998). Also, administration of NT-3 downregulates NPY immunostaining in large diameter sensory neurons (O'Hara et al. 1995; Verge et al. 1996; Sterne et al. 1998), and LIF influences the expression of galanin on small diameter neurons (Corness et al. 1996; Sun & Zigmond, 1996; Thompson et al. 1998).

At present, there is no recognised role for BDNF regulation of expression on any known peptide or neuronal marker. BDNF is synthesised in adult primary sensory neurons (Ernfors et al. 1990; Cho et al. 1997), and a generalised autocrine role of BDNF on sensory neurons has been suggested following in vitro experiments (Achenson et al. 1995). However, in vivo BDNF mRNA is mainly expressed in trkA neurons (Kashiba et al. 1997) and only occasionally in trkB cells, which would not support an autocrine role for this neurotrophin (Michael et al. 1997). Recently, it has been shown that NGF increases BDNF synthesis in almost 90% trkA-expressing DRG cells (Cho et al. 1997; Michael et al. 1997). Similarly a BDNF increase is found in many trkA DRG neurons following peripheral inflammation (Cho et al. 1997), possibly as a response to peripheral NGF, which acts as mediator in inflammation and pain processing (McMahon et al. 1995; McMahon, 1996; Woolf 1996). BDNF is transported axonally to the central and peripheral processes of the trkA neurons (Zhou & Rush, 1996; Michael et al. 1997), where it is found in dense-cored vesicles (Michael et al. 1997). Axotomy increases the synthesis and anterograde transport of BDNF from sensory neurons (James et al. 1998) and it has been postulated that it might act as an anterograde trophic messenger, being released in the dorsal horn under the influence of NGF and modulating nociceptive signalling (Michael et al. 1997). However, a small percentage of trkC neurons and some IB4 labelled small cells also express BDNF mRNA, and NGF treatment does not have any effect on BDNF synthesis in these cells (Michael et al. 1997), which leaves the question of BDNF role in DRG still open.

Following nerve axotomy, GGF mRNA increases rapidly in dorsal root ganglia neurons, coinciding with the beginning of Schwann cell proliferation in the distal nerve stump (Li et al. 1997). Expression of erbB2 and erbB3 receptors on distal nerve stump glial cells is also upregulated initially after injury (Li et al. 1997), and this increase persists in response to GGF, indicating a coordinated response to axotomy between axon and Schwann cells (Carroll et al. 1997). Another example of this interrelationship is the rapid apoptotic death of Schwann cells at neuromuscular junctions following axotomy, which is prevented by injection of GGF (Trachtenberg et al. 1996).

Motoneurons

Nerve axotomy produces a progressive loss of choline acetyltransferase and increased expression of trkB and p75 in motoneurons (Friedman et al. 1995), although the upregulation of p75 has been related to axonal regeneration rather than to a signal of nerve damage (Rende et al. 1993). The changes are particularly evident in brainstem motoneurons, compared with spinal motoneurons (Rende et al. 1997), possibly due to the different responsiveness of the motoneurons to different growth factors released peripherally.

BDNF has been shown to reduce motoneuron death after axotomy in neonatal animals (Yan et al. 1992) and in adults after ventral root avulsion (Novikov et al. 1995; Novikova et al. 1997). Intrathecal administration of BDNF reverses both soma atrophy and reduction of choline acetyl transferase in motoneurons (Kishino et al. 1997). Similar beneficial effects were also observed after BDNF administration to the axotomised hypoglossal (Wang et al. 1997) and sciatic (Friedman et al. 1995) nerves, although the effect of BDNF appears to be dose dependent (Vejsada et al. 1994,1995).

CNTF has a potent trophic role on motoneurons during development and postnatally (Sendtner et al. 1990; Richardson, 1994) and recently a direct effect of CNTF has also been shown on adult motoneuron survival in vivo (Sendtner et al. 1997). Following nerve injury, CNTF in Schwann cells of the distal stump is reduced considerably, this reduction extending to the neuromuscular junction (Hiruma et al. 1997). This deficit might explain the beneficial effect of CNTF administration on target organ motor reinnervation. Indeed, systemic CNTF administration enhances muscle fibre reinnervation and intramuscular nerve branching (Ulenkate et al. 1994), while promoting the preservation of muscle mass (Newman et al. 1996). CNTF also stimulates increased

myelination of regenerating axons (Sahenk et al. 1994), but without any significant effect on the regeneration of proximal axons (Ulenkate et al. 1994).

As CNTF and LIF utilise common signalling pathways (Ip et al. 1992) and have overlapping actions on peripheral neurons, it has been suggested they may have a common mechanism of action. Both CNTF and LIF show increased peripheral retrograde transport after nerve injury (Curtis et al. 1993, 1994). The fact that LIF mRNA is increased in peripheral nerve after axotomy (Sun et al. 1996) and that it is not readily taken up by intramuscular terminals (Curtis et al. 1994) has led to the suggestion that the source of LIF may not be the target muscle but Schwann cells, as for CNTF. Indeed, an upregulation of LIF mRNA can be induced in cultured Schwann cells (Matsuoka et al. 1997), and LIF administration to the axotomised nerve enhances nerve regeneration and myelination, while also increasing muscle mass and muscle contraction force (Tham et al. 1997) Consistently in LIF knockout mice, muscle regeneration is significantly reduced, but counteracted by LIF infusion (Kurek et al. 1997).

NT-3 mRNA is present in significant amounts in adult skeletal muscle (Griesbeck et al. 1995), exerting a trophic role for sensory neurons innervating muscle spindles and for motoneurons (Ernfors et al. 1994; Tessarolo et al. 1994). The strong trophic effect on muscle sensory afferents is evident following exogenous administration of NT-3, even in the absence of the target organ (Oakley et al. 1997). The data from NT-3 knock-out mouse studies have also shown that NT-3 is trophic for proprioceptive and mechanoreceptive sensory neurons (Ernfors et al. 1994; Airaksinen et al. 1996). Consistently, NT-3 containing neurons are mainly localised in trigeminal, cervical and lumbar spinal ganglia (Zhou & Rush, 1995) and in vitro NT-3 promotes neurite outgrowth from DRGs which innervate limb muscles (Hory-Lee et al. 1993). The trophic action of NT-3 on motoneurons is confirmed by the enhanced neurite outgrowth from spinal cord explants as well as by the increased number of motor end-plates formed in cocultures of neurons and muscle (Braun et al. 1996). Furthermore, the exogenous administration of NT-3 restores muscle mass and it is selectively beneficial for the reinnervation of type 2b fast muscle fibres (Sterne et al. 1997*b*), suggesting that different growth factors might sustain preferentially different type of motoneurons, as classified by the phenotype they confer to muscle fibres.

Evidence that GDNF mRNA expression is regulated in skeletal muscle (Henderson et al. 1994) and that it is transported retrogradely to motoneurons (Yan et al. 1995) are strong indications of its physiological role for motoneurons. GDNF is a potent survival factor for motoneurons in vitro and in vivo (Henderson et al. 1994; Yan et al. 1995). Following ventral root avulsion in adults, GDNF administration prevents motoneuron cell death (Li et al. 1995) and shows a long-term effect in reducing axotomy-induced soma atrophy (Matheson et al. 1997). In addition, in adult motoneurons GDNF prevents the axotomy-induced ChAT immunoreactivity decrease (Henderson et al. 1994; Yan et al. 1995). Overexpression of muscle GDNF by transgenic mice increases the number of motor axons innervating the neuromuscular junction in a dose-related manner (Nguyen et al. 1998), although overexpression of GDNF by glial cells or of NT-3 and NT-4 by muscle does not have the same effect. The differential effect of glial and muscle-derived GDNF and the identification of 2 alternatively spliced forms of GDNF from muscle and Schwann cells (Springer et al. 1995) raises the intriguing possibility that both the type of growth factor and the site of synthesis might lead to specific and well defined effects on the recipient neurons. This might be due to the differential availability of the trophic factor and its receptors. For example, in normal adult mice GDNF and GFRα-1 mRNAs are expressed at detectable levels in peripheral nerve, while expression of Ret mRNA is found in spinal motoneurons but not peripherally. After sciatic nerve crush, there is a rapid upregulation of GDNF and GFRα-1 synthesis in distal nerve, with a delayed GDNF mRNA increase in the muscle. This coincides with a rapid increase of Ret mRNA expression in motoneurons and DRG (Naveilhan et al. 1997). Hence, following nerve injury, the mode and site of administration of a trophic factor would be crucial to a resulting beneficial effect.

TROPHIC FACTORS AND REGENERATION

Following peripheral nerve injury and axotomy, there is a disruption in the supply of retrogradely transported neurotrophic factors, leading to neuronal cell death and lack of regeneration. This process can be reversed if the neurons regenerate back to peripheral targets, indicating the dependence on target-derived neurotrophic factors (Mendell, 1996). Hence it seems feasible that the addition of exogenous trophic factors to the microenvironment of the injured nerve might produce results similar to those of target organ reinnervation.

Immediately after a nerve lesion, there is a decrease of trkA and p75 expression in DRG (Krekoski et al. 1996). The ability of NGF to regulate differentially the neuropeptide expression in sensory neurons is paralleled by its capacity in counteracting the effect of nerve injury on the trkA-expressing DRG cells (Verge et al. 1996). Consistently, administration of exogenous NGF has resulted in sustained axonal regeneration (Lindsay 1988; Rich et al. 1989*a*; Hollowell et al. 1990; Chongliang et al. 1992; Derby et al. 1993; Whitworth et al. 1995, 1996), which has been related to a reduction in the incidence of neuronal cell death (Rich et al. 1989*b*). Exogenous NGF enhances regeneration of trkA expressing neurons, but also promotes the regeneration of large myelinated fibres and motor axons, which normally do not express trkA (Chongliang et al. 1992), leading to the suggestion that NGF might be a general promoter of nerve regeneration. Also, it has been shown that continuous intrathecal infusion of NGF may actually delay the onset of regeneration, without compromising the rate of regeneration (Gold, 1997), possibly indicating that the initial loss of NGF following injury might act as signal for the onset of nerve regeneration. While exogenous NGF may facilitate regeneration, the availability of endogenous NGF appears to be essential for collateral sprouting of cutaneous sensory nerves (Diamond et al. 1992*a*). However, neutralisation of endogenous NGF with specific antibodies does not prevent axonal regeneration (Rich et al. 1984; Diamond et al. 1992*b*), showing a considerable difference in the biological response of the peripheral nervous system to contrasting growth stimuli.

Intrathecal administration of NT-3 contributes to the recovery of its specific receptor trkC and of p75 expression in DRG (Verge et al. 1996). Furthermore, delivery to the axotomised nerve decreases the upregulation of NPY-immunoreactive cells in DRG which is seen after axotomy (O'Hara et al. 1995; Verge et al. 1996; Sterne et al. 1998). NT-3 stimulates neurite outgrowth from the stumps of cultured adult DRG (Edström et al. 1996) and in vivo targeted administration of NT-3 to the cut sciatic nerve using fibronectin mats improves the rate and amount of axonal regeneration (Sterne et al. 1997*a*). Following axotomy, continuous infusion of NT-3 has proved to be effective in restoring sensory and motor conduction velocity in dose-dependent manner (Munson & McMahon, 1997), consistently with the neuroprotective role of NT-3 in sensory neuropathy (Gao et al. 1995; Helgren et al. 1997). It is noteworthy that the concentration of NT-3 which shows a beneficial effect on nerve regeneration is much higher than that required for NGF-enhanced regeneration (Munson et al. 1997; Sterne et al. 1997*a*). Axonal regeneration appears to respond quickly to NGF administration (Whitworth et al. 1996), while there is some delay between administration and evidence of an NT-3 effect on nerve regeneration (Sterne et al. 1997*a*) This may be due to slower retrograde axonal transport in large-size sensory neurons compared with small ones (Grant et al. 1979) and to the differential rate of retrograde neurotrophin transport (DiStefano et al. 1992). Also NT-3 appears to promote the regeneration of various subpopulations of DRG neurons (Sterne et al. 1997*a*), possibly due to NT-3 cross-reactivity with trkA and trkB receptors (Squinto et al. 1991; Davies et al. 1995; Rydén & Ibáñez, 1996).

BDNF administration by osmotic pump after sciatic nerve transection produces an improvement in diameter and myelin thickness in regenerating axons in the long term, an effect which is enhanced by combined administration with CNTF (Lewin et al. 1997). Exogenous BDNF administration has also been shown to improve functional recovery following sciatic nerve transection (Lewin et al. 1997), particularly when it is administered directly to the cut nerve (Utley et al. 1996). Similarly, CNTF uptake and retrograde transport to DRG is more rapid following direct injection at the nerve injury site (Curtis et al. 1993). CNTF in peripheral nerve is localised to Schwann cells, and its synthesis is decreased during wallerian degeneration (Friedman et al. 1992). This decrease extends to Schwann cells of intramuscular nerves and neuromuscular junctions (Hiruma et al. 1997), it is very rapid and it is maintained for over 6 mo in cases of chronic denervation (Anand et al. 1997). Nerve regeneration is accompanied by an increase of CNTF, which is indicative that axonal contact with glial cells is crucial to the maintenance of CNTF levels (Friedman et al. 1992; Anand et al. 1997). This is consistent with the experimental findings in mice which exhibit delayed wallerian degeneration, where the expected decrease of CNTF is also delayed for many days (Subang et al. 1997).

Evidence that GDNF might directly affect the peripheral nerve regeneration process are still forthcoming. GDNF has a beneficial effect on axonal regeneration as assessed by the nerve pinch test (Naveilhan et al. 1997) and intrathecal administration of GDNF reverses the decrease in IB4 labelling found in dorsal root ganglia after axotomy (Molliver et al. 1997; Bennett et al. 1998). GDNF also improves the conduction velocity of motoneurons following regeneration (Munson & McMahon, 1997), and that of small diameter sensory neurons (Bennett et al. 1998).

Despite the beneficial effect of GGF on Schwann cell motility and proliferation, as yet there are no data showing whether these effects may indirectly benefit axonal regeneration. However, the expression of c-erbB receptors on Schwann cells in denervated distal stump is rapidly upregulated and is correlated with the increased expression of GGF in response to axotomy (Carroll et al. 1997; Li et al. 1997). In the long-term, there is a time-related downregulation of both c-erbB and p75 receptors on Schwann cells if axonal regeneration is prevented, although GGF synthesis is maintained (Li et al. 1997; You et al. 1997). These results underline the axonal-glial interdependence after nerve injury, as the persistence of responsive Schwann cells in the distal stump is dependent on axonally derived trophic signalling, and in turn the responsive Schwann cells provide trophic support for the regrowing axons.

Although several experiments have demonstrated the usefulness of specific trophic factors in nerve regeneration, it is becoming more apparent that the survival of primary sensory neurons and motoneurons depends on multiple neurotrophic factors acting synergistically or in a well defined sequence. For example, NT-3 and BDNF have opposing regulatory roles on cortical dendritic cells (McAllister et al. 1997) and although such an effect has not been demonstrated for neurons projecting peripherally, it is conceivable that similarity might also be found in the peripheral nervous system. In culture of spiral ganglion cells, the effect of NT-3 is enhanced by LIF administration (Mazzarella et al. 1997). In vitro evidence have also shown that GDNF from Schwann cells and muscle-derived cardiotrophin (CT-1) have a synergistic effect on motoneuron survival. In vivo motoneurons coexpress the receptor for both trophic factors, indicating that an integration of the signal from these 2 factors is essential for motoneuron survival (Arce et al. 1998).

DELIVERY OF NEUROTROPHIC FACTORS

Neurotrophic factors have been advocated as ideally suited for the treatment of neurodegenerative diseases of the CNS, neuropathies and peripheral nerve injury (Anand, 1996; Lindsay, 1996*b*). Many experiments outlined above have made use of miniosmotic pumps for intrathecal delivery of growth factors, in order to maintain a suitable supply for a sufficient length of time. However, this delivery system may not be generally suited for clinical application, and other ways of delivery have been investigated, including systemic delivery by subcutaneous or intraperitoneal injection, and targeted administration to the cut nerve using bioresorbable materials (i.e. fibronectin mats or collagen).

Direct administration of growth factors might be problematic when applied to patients, particularly when a continuous infusion of the molecule is required. It is likely that in future treatment strategies will be based on the transfer of genes, molecules or cells into the nervous system. New molecular biology techniques allow the insertion of specific genes into nondividing neuronal cells, which would then produce their own 'trophic support' molecule (Hoffer & Olson, 1997). Alternatively, regeneration of neurons may be prompted and sustained by grafts of cells which have been genetically manipulated to produce growth factor(s). Recently, genetically modified fibroblasts encapsulated in porous membrane have been shown to be an efficient way of delivering CNTF in a primate model of Huntington's disease (Emerich et al. 1997). A similar approach has also been used to deliver NGF and BDNF (Emerich et al. 1994; Tuszynski et al. 1994, 1996; Kim et al. 1996) and it is conceivable that it would also be applicable to peripheral nerve injury. Growth factor cDNA can also be spliced into defective viruses which act as vectors. Herpes simplex virus expressing NGF can be injected directly into the nerve and transported retrogradely to mimic the physiological role of the neurotrophin. This method has been shown to prevent the decrease of TH activity seen in sympathetic ganglia after axotomy (Federoff et al. 1992). Similarly, administration of CNTF and BDNF transduced in adenoviruses promotes the survival of neonatally axotomised motoneurons (Gravel et al. 1997). Although these experimental strategies are still in the early stages of development, they show the potential of genetic manipulation for therapeutic application in the peripheral and central nervous system. Furthermore, these experiments demonstrate the possibility of modifying neuronal physiology in vivo by augmenting the expression and delivery of a critical gene product to neural cells when extra requirements for growth factors are needed.

REFERENCES

ACHESON A, BARKER PA, ALDERSON RF, MILLER FD, MURPHY RA (1991) Detection of brain-derived neurotrophic factor-like activity in fibroblasts and Schwann cells: inhibition by antibodies to NGF. *Neuron* **7**, 265–275.

ACHESON A, CONOVER JC, FANDL JP, DE CHIARA TM, RUSSELL M, THADANI A et al. (1995) A BDNF autocrine loop in adult sensory neurons prevents cell death. *Nature* **374**, 450–452.

ADLER R (1993) Ciliary neurotrophic factor as an injury factor. *Current Opinion in Neurobiology* **3**, 785–789.

AIRAKSINEN MS, KOLZENBURG M, LEWIN GR, MASU Y, HELBIG C, Wolf E et al. (1996) Specific subtypes of cutaneous mechanoreceptors require neurotrophin-3 following peripheral target innervation. *Neuron* **16**, 287–295.

ANAND P (1996) Neurotrophins and peripheral neuropathy, *Philosophical Transactions of the Royal Society of London [Biological Sciences]* **351**, 449–454.

ANAND P, TERENGHI G, WARNER G, KOPELMAN P, WILLIAMS-CHESTNUT RE, SINICROPI DV (1996) The role of endogenous nerve growth factor in human diabetic neuropathy. *Nature Medicine* **2**, 703–707.

ANAND P, TERENGHI G, BIRCH R, WELLMER A, CEDARBAUM JM, LINDSAY RM et al. (1997) Endogenous NGF and CNTF levels in human peripheral nerve injury. *Neuroreport* **8**, 1935–1938.

ANTON ES, WESKAMP G, REICHARDT LF, MATTHEW WD (1994) Nerve growth factor and its low affinity receptor promote Schwann cell migration. *Proceedings of the National Academy of Sciences of the USA* **91**, 2795–2799.

ARAKAWA Y, SENDTNER M, THOENEN H (1990) Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurons in culture: comparison with other neurotrophic factors and cytokines. *Journal of Neuroscience* **10**, 3507–3515.

ARCE V, POLLOCK RA, PHILIPPE JM, PENNICA D, HENDERSON CE, DELAPEYRIERE O (1998) Synergistic effects of Schwann- and muscle-derived factors on motoneuron survival involve GDNF and cardiotrophin-1 (CT-1). *Journal of Neuroscience* **18**, 1440–1448.

ARDVIDSSON J, YGGE J, GRANT G (1986) Cell loss in lumbar dorsal root ganglia and transganglionic degeneration after sciatic nerve resection in the rat. *Brain Research* **373**, 15–21.

BALOH RH, TANSEY MG, GOLDEN JP, CREEDON DJ, HEUCKEROTH RO, KECK CL et al. (1997) TrnR2, a novel receptor that mediates neurturin and GDNF signalling through Ret. *Neuron* **18**, 793–802.

BANDTLOW CE, HEUMANN R, SCHWAB ME, THOENEN H (1987) Cellular localisation of nerve growth factor synthesis by in situ hybridisation. *EMBO Journal* **6**, 891–899.

BARBACID M (1994) The trk family of neurotrophin receptors. *Journal of Neurobiology* **25**, 1386–1403.

BARDE YA (1990) The nerve growth factor family. *Progress In Growth Factor Research* **2**, 237–248.

BARDE YA (1994) Neurotrophic factors: an evolutionary perspective. *Journal of Neurobiology* **25**, 1329–1333.

BARDE YA, Lindsay RM, Monard D, Thoenen H (1978) New factor released by cultured glioma cells supporting survival and growth of sensory neurons. *Nature* **274**, 818.

BELLIVEAU DJ, KRIVKO I, KOHN J, LACHANCE C, POZNIAK C, RUSAKOV D et al. (1997) NGF and neurotrophin-3 both activate trkA on sympathetic neurons but differentially regulate survival and neuritogenensis. *Journal of Cell Biology* **136**, 375–388.

BENNETT DL, DMIETRIEVA N, PRIESTLEY JV, CLARY D, MCMAHON SB (1996) trkA, CGRP and IB4 expression in retrogradely labelled cutaneous and visceral primary sensory neurons in rat. *Neuroscience Letters* **206**, 33–36.

BENNETT DLH, MICHAEL GJ, RAMACHANDRAN N, MUNSON JB, AVERILL S, YAN Q et al. (1998) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *Journal of Neuroscience* **18**, 3059–3072.

BERKEMEIER LR, WINSLOW JW, KAPLAN DR, NIKOLICS K, GOEDDEL DV, ROSENTHAL A (1991) Neurotrophin-5: a novel neurotrophic factor that activates trkB. *Neuron* **7**, 857–866.

BEUCHE W, FRIEDE RL (1984) The role of non-resident cells in Wallerian degeneration. *Journal of Neurocytology* **13**, 767–796.

BIXBY JL, LILIEN J, REICHARDT LF (1988) Identification of the major proteins that promote neuronal process outgrowth on Schwann cells in vitro. *Journal of Cell Biology* **107**, 353–361.

BRAUN S, CROIZAT B, LAGRANGE MC, WARTER JM, POINDRON P (1996) Neurotrophins increase motoneurons ability to innervate skeletal muscle fibres in rat spinal cord-human muscle cocultures. *Journal of the Neurological Sciences* **136**, 17–23.

BRUSHART TM (1988) Preferential reinnervation of motor nerves by regenerating motor axons. *Journal of Neuroscience* **8**, 1026–1031.

BRUSHART TM (1993) Motor axons preferentially reinnervate motor pathways. *Journal of Neuroscience* **13**, 2730–2738.

BRUSHART TM, SEILER WA (1987) Selective reinnervation of distal motor stumps by peripheral motor axons. *Experimental Neurology* **97**, 289–300.

BUCHMAN VL, DAVIES AM (1993) Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. *Development* **118**, 989–1001.

BUNGE RP (1980) Some observations on the role of the Schwann cells in peripheral nerve regeneration. In *Nerve Repair and Regeneration—Its Clinical and Experimental Basis* (ed. Jewet McCarroll), p. 58–64. St Louis: Mosby.

BUJ-BELLO A, BUCHMAN VL, HORTON A, ROSENTHAL A, DAVIES AM (1995) GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* **15**, 821–828.

CARRAWAY III KL, BURDEN SJ (1995) Neuregulins and their receptors. *Current Opinion in Neurobiology* **5**, 606–612.

CARROLL SL, MILLER ML, FROHNERT PW, KIM SS, CORBETT JA (1997) Expression of neuregulins and their putative receptors, erbB2 and erbB3, is induced during Wallerian degeneration. *Journal of Neuroscience* **17**, 1642–1659.

CHAO MV, BOTHWELL MA, ROSS AH, KOPROWSKI H, LANAHAN AA, BUCK CR et al. (1986) Gene transfer and molecular cloning of the human NGF receptor. *Science* **232**, 518–521.

CHAO MV, HEMPSTEAD BL (1995) p75 and trk: a two receptor system. *Trends in Neuroscience* **18**, 321–326.

CHEN MS, BERMINGHAM-MCDONOGH O, DANEHY FT, NOLAN C, SCHERER SS, LUCAS J et al. (1994) Expression of multiple neuregulin transcripts in postnatal rat brain. *Journal of Comparative Neurology* **349**, 389–400.

CHO HJ, KIM SY, PARK MJ, KIM DS, KIM JK, CHU MY (1997) Expression of mRNA for brain-derived neurotrophic factor in the dorsal root ganglion following peripheral inflammation. *Brain Research* **749**, 358–362.

CHONGLIANG HE, ZHONGWEI C, ZHENGRONG C (1992) Enhancement of motor regeneration by growth factor. *Microsurgery* **13**, 151–154.

CORNESS J, SHI TJ, XU ZQ, BRULET P, HÖKFELT T (1996) Influence of leukemia inhibitory factor on galanin/GMAP and neuropeptide Y expression in mouse primary sensory neurons after axotomy. *Experimental Brain Research* **112**, 79–88.

CURTIS R, ADRYAN KM, ZHU Y, HARKNESS PJ, LINDSAY RM, DISTEFANO PS (1993) Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. *Nature* **365**, 253–255.

CURTIS R, SCHERER S, SOMOGYI R, ADRYAN KM, IP NY, ZHU Y, et al. (1994) Retrograde axonal transport of LIF is increased by peripheral nerve injury: correlation with increased LIF expression in distal nerve. *Neuron* **12**, 191–204.

DAVIS S, ALDRICH TH, VALENZUELA DM, WONG V, FURTH M, SQUINTO SP et al. (1991) The receptor for ciliary neurotrophic factor. *Science* **253**, 59–63.

DAVIES AM (1996) The neurotrophic hypothesis: where does it stand? *Philosophical Transactions of the Royal Society of London [Biological Sciences]* **351**, 389–394.

DAVIES AM, BANDTLOW C, HEUMANN R, KORSHING S, ROHRER H, THOENEN H (1987) Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. *Nature* **326**, 353–358

DAVIES AM, MINICHIELLO L, KLEIN R (1995) Developmental changes in NT-3 signalling via trkA and trkB in embryonic neurons. *EMBO Journal* **14**, 4482–4489.

DELLON AL (1981) *Evaluation of Sensibility and Re-education of Sensation in the Hand.* Baltimore: Williams and Wilkins.

DERBY A, ENGLEMAN VW, FRIERDICH GE, NEISES G, RAPP SR, ROUFA DG (1993) Nerve growth factor facilitates regeneration across nerve gaps: morphological and behavioural studies in rat sciatic nerve. *Experimental Neurology* **119**, 176–191.

DE VRIES GH (1993) Schwann cell proliferation. In *Peripheral Neuropathy*, 3rd edn (ed. Dyck PJ, Thomas PK, Griffin JW, Low PD, Poduslo J), pp. 290–298. Philadelphia: W. B. Saunders.

DIAMOND J, HOLMES M, COUGHLIN M (1992*a*) Endogenous NGF and nerve impulses regulate the collateral sprouting of sensory axons in the skin of the adult rat. *Journal of Neuroscience* **12**, 1454–1466.

DIAMOND J, FOERSTER A, HOLMES M, COUGHLIN M (1992*b*) Sensory nerves in adult rats regenerate and restore sensory function to the skin independently of endogenous NGF. *Journal of Neuroscience* **12**, 1467–1476.

DISTEFANO PS, FRIEDMAN B, RADZIEJEWSKI C, ALEXANDER C, BOLAND P, SCHICK CM et al. (1992) The neurotrophins BDNF, NT-3 and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. *Neuron* **8**, 983–993.

DONG Z, BRENNAN A, LIU N, YARDEN Y, LEFKOWITZ G, MIRSKY R et al. (1995) Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation and maturation of rat Schwann cell precursors. *Neuron* **15**, 585–596.

DYKES RW (1984) Central consequences of peripheral nerve injury. *Annals in Plastic Surgery* **13**, 412–428.

EDSTRÖM A, EKSTRÖM PAR, TONGE D (1996) Axonal outgrowth and neuronal apoptosis in cultured adult mouse dorsal root ganglion preparations: effects of neurotrophins, of inhibition of neurotrophin actions and of prior axotomy. *Neuroscience* **75**, 1165–1174.

EMERICH DF, HAMMAG JP, BAETGE EE, WINN SR (1994) Implantation of polymer-encapsulated human nerve growth factor-secreting fibroblasts attenuates the behavioral and neuropathological consequences of quinolic acid injections into rodent striatum. *Experimental Neurology* **130**, 141–150.

EMERICH DF, WINN SR, HANTRAYE PM, PESCHANSKI M, CHEN EY, CHUY P et al. (1997) Protective effect of encapsulated cells producing neurotrophic factor CNTF in a monkey model of Huntington's disease. *Nature* **386**, 395–399.

ERIKSSON NP, LINDSAY RM, ALDSKOGIUS H (1994) BDNF and NT-3 rescue sensory but not motoneurons following axotomy in neonate. *NeuroReport* **5**, 1445–1448.

ERNFORS P, IBANEZ CF, EBENDAL T, OLSON L, PERSSON H (1990) Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in brain. *Proceedings of the National Academy of Sciences of the USA* **87**, 5454–5458.

ERNFORS P, MERLIO J, PERSSON H (1992) Cell expressing mRNA for neurotrophins and their receptors during embryonic rat development. *European Journal of Neuroscience* **4**, 1140–1158.

ERNFORS P, ROSARIO CM, MERLIO JP, GRANT G, ALDSKOGIUS H, PERSSON H (1993) Expression of mRNA for neurotrophin receptors in the dorsal root ganglion and spinal cord during development and following peripheral or central axotomy. *Molecular Brain Research* **17**, 217–226.

ERNFORS P, LEE KF, KUCERA J, JAENISCH R (1994) Lack of neurotrophin-3 leads to deficiencies in the peripheral nervou system and loss of limb proprioceptive afferents. *Cell* **77**, 503–51

FEDEROFF HJ, GESCHWIND MD, GELLER AI, KESSLER J (1992) Expression of nerve growth factor in vivo from defective herpes simplex virus 1 vector prevents effects of axoton on sympathetic ganglia. *Proceedings of the National Academy Sciences of the USA* **89**, 1636–1640.

FITZGERALD M, WALL PD, GOEDERT M, EMSON PC (198 Nerve growth factor counteracts the neurophysiological a neurochemical effects of chronic sciatic nerve section. *Bra Research* **332**, 131–141.

FRIEDLANDER DR, GRUMET M, EDELMAN GM (198 Nerve growth factor enhances expression of neuron-glial c adhesion molecule in PC12 cells. *Journal of Cell Biology* **10** 413–419.

FRIEDMAN B, SCHERER SS, RUDGE JS, HELGREN N MORRISEY D, McLAIN J et al. (1992) Regulation of cilia neurotrophic factor expression in myelin-related Schwann cells vivo. *Neuron* **9**, 295–305.

FRIEDMAN B, KLEINFIELD D, IP NY, VERGE VM MOULTON R, BOLAND P et al. (1995) BDNF and NT-4 exert neurotrophic influences on injured adult spinal mot neurons. *Journal of Neuroscience* **15**, 1044–1056.

FUNAKOSHI H, FRIESEN J, BARBANY G, TIMMUSK ZACHIRSSON O, VERGE VM et al. (1993) Different expression of mRNA for neurotrophins and their receptors af axotomy of the sciatic nerve. *Journal of Cell Biology* **1** 455–465.

GAO WQ, DYBDAL N, SHINSKY N, MURNANE A, SCHI ELZER C, SIEGEL M et al. (1995) Neurotrophin-3 rever experimental cisplantin-induced peripheral sensory neuropati *Annals of Neurology* **38**, 30–37.

GARGANO N, LEVI A, ALEMA S (1997) Modulation of ne growth factor internalisation by direct interaction between p and trkA receptors. *Journal of Neuroscience Research* **50**, 1–1

GLASS DJ, YANCOPOULOS GD (1993) The neurotrophins a their receptors. *Trends in Cell Biology* **3**, 262–268.

GOLD BG (1997) Axonal regeneration of sensory nerves is dela by continuous intrathecal infusion of nerve growth fact *Neuroscience* **76**, 1153–1158.

GRANT G, ARVIDSSON J, ROBERTSON B, YGGE J (19 Transganglionic transport of horseradish peroxidase in prim sensory neurons. *Neuroscience Letters* **12**, 23–28.

GRAVEL C, GÖTZ R, LORRAIN A, SENDTNER M (19 Adenoviral gene transfer of ciliary neurotrophic factor brain-derived neurotrophic factor leads to long-term survival axotomised motor neurons. *Nature Medicine* **3**, 765–770.

GRIESBECK O, PARSADANIAN AS, SENDTNER THOENEN H (1995) Expression of neurotrophins in skel muscle: quantitative comparison and significance for mc neuron survival and maintenance of function. *Journal Neuroscience Research* **42**, 21–33.

GROVES MJ, CHRISTOPHERSON T, GIOMETTO B, SCA AVILLI F (1997) Axotomy-induced apoptosis in adult primary sensory neurons. *Journal of Neurocytology* **26**, 615–(

GU X, THOMAS PK, KING RHM (1995) Chemotropism in nc regeneration studied in tissue culture. *Journal of Anatomy* **1** 153–163.

HALL SM (1986) Regeneration in cellular and acellular autogr in the peripheral nervous system. *Neuropathology and App Neurobiology* **12**, 27–46.

HALL SM (1997) Axonal regeneration through acellular mu grafts. *Journal of Anatomy* **190**, 57–71.

HALLBROOK F, IBANEZ CF, PERSSON H (1991) Evolution studies of the nerve growth factor family reveal a novel mem abundantly expressed in Xenopus ovary. *Neuron* **6**, 845–858

HELGREN ME, SQUINTO SP, DAVIS HL, PARRY BOULTOn TG, HECK CS et al. (1994) Trophic effect of cil

neurotrophic factor on denervated skeletal muscle. *Cell* **76**, 493–504.

HELGREN ME, CLIFFER KD, TORRENTO K, CAVNOR C, CURTIS R, DISTEFANO PS et al. (1997) Neurotrophin-3 administration attenuates deficits of pyroxidine-induced large fibre sensory neuropathy. *Journal of Neuroscience* **17**, 372–382.

HENDERSON CE, PHILIPPS HS, POLLOCK RA, DAVIES AM, LEMEULLE C, ARMANINI MP et al. (1994) GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. *Science* **266**, 1062–1064

HEUMANN R, KORSHING S, BANDTLOW C, THOENEN H (1987) Changes of nerve growth factor synthesis in non-neuronal cells in response to sciatic nerve transection. *Journal of Cell Biology* **104**, 1623–1631.

HIMES BT, TESSLER A (1989) Death of some dorsal root ganglion neurons and plasticity of others following sciatic nerve section in adult and neonatal rats. *Journal of Comparative Neurology* **284**, 215–230.

HIRUMA S, SHIMIZU T, HÚRUTA T, SATOU T, HU JG, TANJI K et al. (1997) Ciliary neurotrophic factor immunoreactivity in rat intramuscular nerve during reinnervation through a silicone tube after severing of the rat sciatic nerve. *Experimental Molecular Pathology* **64**, 23–30.

HOFFER B, OLSON L (1997) Treatment strategies for neurodegenerative diseases based on trophic factors and cell transplantation techniques. *Journal of Neural Transmission* **104** (Suppl 49), 1–10.

HÖKFELT T, ZHANG X, WIESENFELD-HALLIN Z (1994) Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends in Neuroscience* **17**, 22–30.

HOLLOWELL JP, VILLADIEGO A, RICH KM (1990) Sciatic nerve regeneration across gaps within silicone chambers: long-term effects of NGF and consideration on axonal branching. *Experimental Neurology* **110**, 45–51.

HOPKINS SJ, ROTHWELL NJ (1995) Cytokines and the nervous system I: expression and recognition. *Trends in Neuroscience* **18**, 83–88.

HORY-LEE F, RUSSELL M, LINDSAY RM, FRANK E (1993) Neurotrophin-3 supports the survival of developing muscle sensory neurons in culture. *Proceeding of the National Academy of Sciences of the USA* **90**, 2613–2617,

IP NY, YANCOPOULOS GD (1992) Ciliary neurotrophic factor and its receptor complex. *Progress in Growth Factors Research* **4**, 139–155.

IP NY, NYE SH, BOULTON TG, DAVIS S, TAGA T, LI Y et al. (1992) CNTF and LIF act on neuronal cells via shared signalling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell* **69**, 1121–1132.

JAMES R, TONRA JR, CURTIS R, WONG V, CLIFFER KD, PARK JS et al. (1998) Axotomy upregulates the anterograde transport and expression of brain derived neurotrophic factor in sensory neurons. *Journal of Neuroscience* **18**, 4371–4383.

JING SQ, WEN DZ, YU YB, HOLST PL, LUO Y, FANG M et al. (1996) GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-α, a novel receptor for GDNF. *Cell* **85**, 1113–1124.

JOHNSON ER Jr, YIP HK (1985) Central nervous system and peripheral nerve growth factor provide trophic support critical to mature sensory neuronal survival. *Nature* **314**, 751–752.

KAPLAN DR, HEMPSTEAD BL, MARTIN-ZANCA D, CHAO M, PARADA LF (1991) The trk protooncogene product: a signal transducing receptor for nerve growth factor. *Science* **252**, 554–558.

KASHIBA H, SENBA E, KAWAI Y, UEDA Y, TOHYAMA M (1992) Axonal blockade induces the expression of vasoactive intestinal peptide and galanin in rat dorsal root ganglion neurons. *Brain Research* **577**, 19–28.

KASHIBA H, UEDA Y, UEYAMA T, NEMOTO K, SENBA E (1997) Relationship between BDNF- and trk-expressing neurons in rat dorsal root ganglion. *Neuroreport* **8**, 1229–1234.

KEREKES N, LANDRY M, RYDH-RYNDER M, HÖKFELT T (1997) The effect of NGF, BDNF and bFGF on expression of galanin in cultured dorsal root ganglia. *Brain Research* **754**, 131–141

KIM DH, GUTIN PH, NOBLE LJ, NATHAN D, YU JS, NOCKELS RP (1996) Treatment with genetically engineered fibroblasts producing NGF or BDNF can accelerate recovery from traumatic spinal cord injury in the adult rat. *Neuroreport* **7**, 2221–2225.

KISHINO A, ISHIGE Y, TATSUNO T, NAKAYAMA C, NOGUCHI H (1997) BDNF prevents and reverses adult rat motor neuron degeneration and induces axonal outgrowth. *Experimental Neurology* **144**, 273–286.

KLEIN R, PARADA LF, COULIER F, BARBACID M (1989) trkB, a novel tyrosine kinase receptor expressed during mouse neural development. *EMBO Journal* **8**: 3701–3709.

KLEIN R, LAMBALLE F, BRYANT S, BARBABCID M (1992) the trkB tyrosine protein kinase is a receptor for neurotrophin-4. *Neuron* **8**, 947–956.

KREKOSKI CA, PARHAD IM, CLARK AW (1996) Attenuation and recovery of nerve growth factor receptor mRNA in dorsal root ganglion following axotomy. *Journal of Neuroscience Research* **43**, 1–11.

KUREK JB, BOWER JJ, ROMANELLA M, KOENTGEN F, MURPHY M, AUSTIN L (1997) The role of leukaemia inhibitory factor in skeletal muscle regeneration. *Muscle & Nerve* **20**, 815–822.

LAMBALLE F, KLEIN R, BARBACID M (1991) trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell* **66**, 967–979.

LEVI-MONTALCINI R (1987) The nerve growth factor: thirty-five years later. *EMBO Journal* **6**, 1145–1154.

LEWIN GR, BARDE YA (1996) Physiology of the neurotrophins. *Annals Review of Neuroscience* **19**, 289–317.

LEWIN SL, UTLEY DS, CHENGET, VERITY AN, TERRIS DJ (1997) Simultaneous treatment with BDNF and CNTF after peripheral nerve transection and repair enhances rate of functional recovery compared with BDNF treatment alone. *Laryngoscope* **107**, 992–999.

LI L, WU W, LIN LF, LEI M, OPPENHEIM RW, HOUENOU LJ (1995) Rescue of adult mouse motoneurons from injury-induced cell death by glial cell line-derived neurotrophic factor. *Proceedings of the National Academy of Sciences of the USA* **92**, 9771–9775.

LI H, TERENGHI G, HALL SM (1997) Effects of delayed re-innervation on the expression of c-erbB receptors by chronically denervated rat Schwann cells in vivo. *Glia* **20**, 333–347.

LIN LH, DOHERTY DH, LILE JD, BEKTESH S, COLLINS F (1993) GDNF: a glial cell-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260**, 1130–1132.

LINDSAY RM (1988) Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. *Journal of Neuroscience* **8**, 2394–2405.

LINDSAY RM (1996*a*) Role of neurotrophins and trk receptors in the development and maintenance of sensory neurons. An overview. *Philosophical Transactions of the Royal Society of London [Biological Sciences]* **351**, 365–373.

LINDSAY RM (1996*b*) Therapeutic potential of the neurotrophins and neurotrophin-CNTF combinations in peripheral neuropathies and motor neuron disease. In *Growth Factors as Drugs for Neurological and Sensory Disorders (Ciba Foundation Symposium 196)*, pp. 39–53. Chichester: Wiley.

LINDSAY RM, HARMAR AJ (1989) Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. *Nature* **337**, 362–364.

LISS AG, AF EKENSTAM FW, WIBERG M (1994) Cell loss in sensory ganglia after peripheral nerve injury. An anatomical tracer study using lectin-coupled horseradish peroxidase in cats. *Scandinavian Journal of Plastic Reconstructive Surgery and Hand Surgery* **28**, 117–188.

MADISON RD, ARCHIBALD SJ, BRUSHART TM (1996) Reinnervation accuracy of the rat femoral nerve by motor and sensory neurons. *Journal of Neuroscience* **16**, 5698–5703.

MAHANTHAPPA NK, ANTON ES, MATTHEW WD (1996) Glial growth factor 2, a soluble neuregulin, directly increases Schwann cell motility and indirectly promotes neurite outgrowth. *Journal of Neuroscience* **16**, 4673–4683.

MAISONPIERRE PC, BELLUSCIO L, FRIEDMAN B, ALDERSON RF, WIEGAND SJ, FURTH ME et al. (1990) NT3, BDNF and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. *Neuron* **5**, 501–509

MANESS LM, KASTIN AJ, WEBER JT, BANKS WA, BECKMAN BS, ZADINA JE (1994) The neurotrophins and their receptors: structure, function and neuropathology. *Neuroscience and Biobehavioural Review* **18**, 143–159.

MARCHIONNI MA, GOODEARL ADJ, CHEN MS, BERMINGHAM-MCDONOGH O, KIRK C, HENDRICKS M et al. (1993) Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. *Nature* **362**, 312–318.

MARTINI R, SCHACHNER M, BRUSHART TM (1994) The L2/HNK-1 carbohydrate is preferentially expressed by previously motor axon-associated Schwann cells in reinnervated peripheral nerves. *Journal of Neuroscience* **14**, 7180–7191.

MARZELLA PL, CLARK GM, SHEPHERD RK, BARTLETT PF, KILPATRICK TJ (1997) LIF potentiates the NT-3-mediated survival of spiral ganglia neurones in vitro. *Neuroreport* **8**, 1641–1644.

MATHESON CR, WANG J, COLLINS FD, YAN Q (1997) Long-term survival effects of GDNF on neonatal rat facial motoneurons after axotomy. *Neuroreport* **8**, 1739–1742.

MATSUOKA I, NAKANE A, KURIHARA K (1997) Induction of LIF-mRNA by TGF-beta 1 in Schwann cells. *Brain Research* **776**, 170–180.

MCALLISTER AK, KATZ LC, LO DC (1997) Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth. *Neuron* **18**, 767–778.

MCMAHON SB (1996) NGF as a mediator of inflammatory pain. *Philosophical Transactions of the Royal Society of London [Biological Sciences]* **351**, 431–440.

MCMAHON SB, ARMANINI MP, LING LH, PHILIPPS HS (1994) Expression and coexpression of trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. *Neuron* **12**, 1161- 1171.

MCMAHON SB, BENNETT DLH, PRIESTLEY JV, SHELTON DL (1995) The biological effects of endogenous nerve growth factor on adult sensory neurons revealed by trkA-IgG fusion molecule. *Nature Medicine* **1**, 774–780.

MENDELL LM (1996) Neurotrophins and sensory neurons: role in development, maintenance and injury. A thematic summary. *Philosophical Transactions of the Royal Society of London [Biological Sciences]* **351**, 463–467.

MEYER M, MTSUOKA I, WETMORE C, OLSON L, THOENEN H (1992) Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. *Journal of Cell Biology* **119**, 45–54.

MICHAEL GJ, AVERILL S, NITKUNAN A, RATTRAY M, BENNETT DLH, YAN Q et al. (1997) Nerve growth factor treatment increases brain-derived neurotrophic factor selectively in trkA-expressing dorsal root ganglion cells and in their central terminations within the spinal cord. *Journal of Neuroscience* **17**, 8476–8490.

MOLLIVER DC, WRIGHT DE, LEITNER ML, PARSADANIAN ASH, DOSTER K, WEN D et al. (1997) IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron* **19**, 849–861.

MULDERRY PK (1994) Neuropeptide expression by newborn and adult rat sensory neurons in culture: effect of nerve growth factor and other neurotrophic factors. *Neuroscience* **59**, 673–688.

MUNSON JB, MCMAHON SB (1997) Effects of GDNF on axotomised sensory and motor neurons in adult rats. *European Journal of Neuroscience* **9**, 1126–1129.

MUNSON JB, SHELTON DL, MCMAHON B (1997) Adult mammalian sensory and motor neurons: roles of endogenous neurotrophins and rescue by exogenous neurotrophin after axotomy. *Journal of Neuroscience* **17**, 470–476.

NAVEILHAN P, ELSHAMY VM, EMFORS P (1997) Differential regulation of mRNAs for GDNF and its receptors Ret and GDNFα after sciatic nerve lesion in the mouse. *European Journal of Neuroscience* **9**, 1450–1460.

NAWA H, YAMAMORI T, LE T, PATTERSON PH (1990) Generation of neuronal diversity: analogies and homologies with hematopoiesis. *Cold Spring Harbour Symposium of Quantitative Biology* **55**, 247–253.

NEWMAN JP, VERITY AN, HAWATMEH S, FEE WE Jr, TERRIS DJ (1996) Ciliary neurotrophic factor enhances peripheral nerve regeneration. *Head and Neck Surgery* **122**, 399–403

NOVIKOV L, NOVIKOVA L, KELLERTH JO (1995) Brain derived neurotrophic factor promotes survival and blocks nitric oxide synthase expression in adult spinal motoneurons after ventral root avulsion. *Neuroscience Letters* **200**, 45–48.

NOVIKOVA L, NOVIKOV L, KELLERTH JO (1997) Effects o neurotransplants and BDNF on the survival and regeneration o injured adult spinal motoneurons. *European Journal of Neuro science* **9**, 2774–2777.

NGUYEN QT, PARSADANIAN AS, SNIDER WD, LICH TMAN JW (1998) Hyperinnervation of neuromuscular junction caused by GDNF overexpression in muscle. *Science* **279** 1725–1729.

OAKLEY RA, GARNER AS, LARGE TH, FRANK E (1995 Muscle sensory neurons require neurotrophin-3 from peripher tissues during the period of normal cell death. *Development* **121** 1341–1350.

OAKLEY RA, LEFCORT FB, CLARY DO, REICHARDT LI PREVETTE D, OPPENHEIM RW et al. (1997) Neurotrophin 3 promotes the differentiation of muscle spindle afferents in th absence of peripheral targets. *Journal of Neuroscience* **1** 4262–4274.

O'HARA S, TANTUWAYA V, DI STEFANO PS, SCHMID RE (1995) Exogenous NT-3 mitigates the transganglion neuropeptide Y response to sciatic nerve injury. *Brain Researc* **699**, 143–148.

OPPENHEIM RW (1991) Cell death during development of tl nervous system. *Annual Reviews of Neuroscience* **14**, 453–501.

PERRY VH, BROWN MC, GORDON S (1987) The macropha response to central and peripheral nerve injury. *Journal Experimental Medicine* **165**, 1218–1223.

PURVES D (1986) The trophic theory of neural connections. *Tren in Neuroscience* **9**, 486–489.

RATH S, GREEN CJ (1991 *a*) Selectivity of distal reinnervation regenerating mixed motor and sensory nerve fibres across mus grafts in rats. *British Journal of Plastic Surgery* **44**, 215–218.

RATH S, GREEN CJ (1991 *b*) Lack of topographical specificity sensory nerve regeneration through muscle grafts in rats. *Jour of Hand Surgery* **16B**, 524–530.

RENDE M, MUIR D, RUOSLAHTI E, HAGG T, VARON MANTHORPE M (1992) Immunolocalisation of ciliary ne otrophic factor in adult rat sciatic nerve. *Glia* **5**, 25–32.

RENDE M, PROVENZANO C, TONALI P (1993) Modulation low-affinity nerve growth factor receptor in injured adult

spinal cord motoneurons. *Journal of Comparative Neurology* **338**, 560–574.

RENDE M, PROVENZANO C, STIPA G, TONALI P (1997) Effects of sciatic nerve grafts on choline acetyltransferase and p75 expression in transected adult hypoglossal motoneurons. *Neuroscience* **81**, 517–525.

REYNOLDS ML, WOOLF CJ (1993) Reciprocal Schwann cell-axon interaction. *Current Opinion in Neurobiology* **3**, 683–693.

RICH KM, YIP HK, OSBORNE PA, SCHMIDT RE, JOHNSON EM (1984) Role of nerve growth factor in the adult dorsal root ganglion neuron and its response to injury. *Journal of Comparative Neurology* 230, 110–118.

RICH KM, ALEXANDER TD, PRYOR JC, HOLLOWELL JP (1989*a*) Nerve growth factor enhances regeneration through silicone chambers. *Experimental Neurology* **105**, 162–170.

RICH KM, DISCH SP, EICHLER ME (1989*b*) The influence of regeneration and nerve growth factor on the neuronal cell body reaction to injury. *Journal of Neurocytology* **18**, 569–576.

RICHARDSON PM (1994) Ciliary neurotrophic factor: a review. *Pharmacological Therapy* **63**, 187–198.

ROBERTSON MD, TOEWS AD, BOULDIN TW, WEAVER J, GOINES ND, MORELL P (1995) NGFR-mRNA expression in sciatic nerve: a sensitive indicator of early stages of axonopathy. *Molecular Brain Research* **28**, 231–238.

RODRIGUEZ-TEBAR A, DECHANT G, BARDE YA (1990) Binding of brain-derived neurotrophic factor to nerve growth factor receptor. *Neuron* **4**, 487–492.

RODRIGUEZ-TEBAR A, DECHANT G, GOTZ R, BARDE YA (1992) Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO Journal* **11**, 917–922.

ROTHWELL NJ, HOPKINS SJ (1995) Cytokines and the nervous system II: actions and mechanisms of action. *Trends in Neuroscience* **18**, 130–136.

RUSH RA, MAYO R, ZETTLER C (1995) The regulation of nerve growth factor synthesis and delivery to peripheral neurons. *Pharmacological Therapy* **65**, 93–123.

RUSH RA, CHIE E, LIU D, TAFRESHI A, ZETTLER C, ZHOU XF (1997) Neurotrophic factors are required by mature sympathetic neurons for survival, transmission and connectivity. *Clinical and Experimental Pharmacology and Physiology* **24**, 549–555.

RUTISHAUSER U (1993) Adhesion molecules of the nervous system. *Current Opinion in Neurobiology* **3**, 709–715.

RYDÉN M, IBÁÑEZ CF (1996) Binding of neurotrophin-3 to $p75^{LNGFR}$, trkA and trkB mediated by a single functional epitope distinct from that recognised by trkC. *Journal of Biological Chemistry* **271**, 5623–5627.

SAHENK Z, SEHARASEYON J, MENDELL JR (1994) CNTF potentiates peripheral nerve regeneration. *Brain Research* **655**, 246–250.

SALZER JL, BUNGE RP (1980) Studies of Schwann cell proliferation. I. An analysis in tissue culture of proliferation during development, Wallerian degeneration and direct injury. *Journal of Cell Biology* **84**, 739–752.

SALZER JL, WILLIAMS AK, GLASER L, BUNGE RP (1980) Studies of Schwann cell proliferation. II. Characterisation of the stimulation and specificity of the response to a neurite membrane fraction. *Journal of Cell Biology* **84**, 753–766.

SCHMALBRUCH H (1987) Loss of sensory neurons after sciatic nerve sections in the rat. *Anatomical Record* **219**, 323–329.

SEILHEIMER B, SCHACHNER M (1987) Regulation of neural cell adhesion molecule expression on cultured mouse Schwann cells by nerve growth factor. *EMBO Journal* **6**, 1611–1616.

SENDTNER M, KREUTZBERG GW, THOENEN H (1990) Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature* **345**, 440–441.

SENDTNER M, STOCKLI KA, THOENEN H (1992) Synthesis and localisation of ciliary neurotrophic factor in the sciatic nerve of the adult rat after lesion and during regeneration. *Journal of Cell Biology* **118**, 139–148.

SENDTNER M, GOTZ R, HOLTMANN B, THOENEN H (1997) Endogenous ciliary neurotrophic factor is a lesion factor for axotomised motoneurons in adult mice. *Journal of Neuroscience* **17**, 6999–7006.

SNIDER WD, SILOS-SANTIAGO I (1996) Dorsal root ganglion neurons require functional neurotrophin receptors for survival during development. *Philosophical Transactions of the Royal Society of London [Biological Sciences]* **351**, 393–404.

SON YL, THOMPSON WJ (1995) Schwann cell processes guide regeneration of peripheral axons. *Neuron* **14**, 125–132.

SPRINGER JE, SEEBURGER JL, HE J, GABREA A, BLANKENHORN EP, BERGMAN LW (1995) cDNA sequence and differential mRNA regulation of two forms of glial cell-line derived neurotrophic factor in Schwann cells and rat skeletal muscle. *Experimental Neurology* **131**, 47–52.

SQUINTO SP, STITT TN, ALDRICH TH, DAVIS S, BIANCO SM, RADZIEJEWSKI C et al. (1991) trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3, but not nerve growth factor. *Cell* **65**, 885–893.

STÉRNE GD, BROWN RA, GREEN CJ, TERENGHI G (1997*a*) Neurotrophin-3 delivered locally via fibronectin mats enhances peripheral nerve regeneration. *European Journal of Neuroscience* **9**, 1388–1396.

STERNE GD, COULTON GR, BROWN RA, GREEN CJ, TERENGHI G (1997*b*) Neurotrophin-3 enhanced nerve regeneration selectively improves recovery of muscle fibres expressing myosin heavy chains 2b. *Journal of Cell Biology* **139**, 709–715.

STERNE GD, BROWN RA, GREEN CJ, TERENGHI G (1998) NT-3 modulates NPY expression in primary sensory neurons following peripheral nerve injury. *Journal of Anatomy*, in press.

STOCKLI KA, LOTTSPEICH F, SENDTNER M, MASIAKOWSKI P, CARROLL P, GOTZ R et al. (1989) Molecular cloning expression and regional distribution of rat ciliary neurotrophic factor. *Nature* **342**, 920–923.

SUBANG MC, BISBY MA, RICHARDSON PM (1997) Delay of CNTF decrease following peripheral nerve injury in C57BL/Wld mice. *Neuroscience Research* **49**, 563–568.

SUN Y, LANDIS SC, ZIGMOND RE (1996) Signals triggering the induction of leukaemia inhibitory factor in sympathetic superior cervical ganglia and their nerve trunks after axonal injury. *Molecular and Cellular Neuroscience* **7**, 152–163.

SUN Y, ZIGMOND RE (1996) Leukaemia inhibitory factor induced in the sciatic nerve after axotomy is involved in the induction of galanin in sensory neurons. *European Journal of Neuroscience* **8**, 2213–2220.

TESSAROLO L, VOGEL KS, PALKO ME, REID SW, PARADA LF (1994) Targeted mutation in the neurotrophin-3 gene results in loss of muscle sensory neurons. *Proceedings of the National Academy of Sciences of the USA*, **91**, 11844–11848.

TERENGHI G, CALDER JS, BIRCH R, HALL S (1998) A morphological study of Schwann cells and axonal regeneration in chronically transected human peripheral nerves. *Journal of Hand Surgery* **23B**, in press.

TERZIS JK, SMITH KL (1990) Repair and grafting of the peripheral nerve. In *General Principles of Plastic Surgery* (ed. McCarthy JG), pp. 630–697. Philadelphia: W. B. Saunders.

THAM S, DOWSING B, FINKELSTEIN D, DONATO R, CHEEMA SS, BARTLETT PF et al. (1997) Leukaemia inhibitory factor enhances the regeneration of transected rat sciatic nerve and the function of reinnervated muscle. *Journal of Neuroscience Research* **47**, 208–215.

THOENEN H, BARDE Y (1980) Physiology of nerve growth factor. *Physiological Reviews* **60**, 1284–1335.

THOMPSON SWN, VERNALLIS AB, HEATH JK, PRIESTLEY

JV (1997) Leukaemia inhibitory factor is retrogradely transported by a distinct population of adult sensory neurons. Co-localisation with trkA and other neurochemical markers. *European Journal of Neuroscience* **9**, 1244–1251.

THOMPSON SWN, PRIESTLEY JV, SOUTHALL A (1998) gp130 cytokines, leukaemia inhibitory factor and interleukin-6 induce neuropeptide expression in intact adult rat sensory neurons in vivo: time-course, specificity and comparison with sciatic nerve axotomy. *Neuroscience* **84**, 1247–1255.

TRACHTENBERG JT, THOMPSON WJ (1996) Schwann cell apoptosis at developing neuromuscular junctions is regulated by glial growth factor. *Nature* **379**, 174–177.

TUSZYNSKI MH, PETERSON DA, RAY J, BAIRD A, NAKAHARA Y, GAGE FH (1994) Fibroblasts genetically modified to produce nerve growth factor induce robust neuritic ingrowth after grafting to the spinal cord. *Experimental Neurology* **126**, 1–14.

TUSZYNSKI MH, GABRIEL K, GAGE FH, SUHR S, MEYER S, ROSSETTI A (1996) Nerve growth factor delivery by gene transfer induces differential outgrowth of sensory, motor and noradrenergic neurites after adult spinal cord injury. *Experimental Neurology* **137**, 157–173.

ULENKATE HJLM, KAAL ECA, GISPEN WH, JENNEKENS FGI (1994) Ciliary neurotrophic factor improves muscle fibre reinnervation after facial nerve crush in young rats. *Acta Neuropathologica (Berlin)* **88**, 558–564.

UTLEY DS, LEWIN SL, CHENG ET, VERITY AN, SIERRA D, TERRIS DJ (1996) Brain derived neurotrophic factor and collagen tubulization enhance functional recovery after peripheral nerve transection and repair. *Archives of Otorhinolaryngology and Head and Neck Surgery* **122**, 407–413.

VEJSADA R, SAGOT Y, KATO AC (1994) BDNF-mediated rescue of axotomised motor neurones decreases with increasing dose. *Neuroreport* **5**, 1889–1892.

VEJSADA R, SAGOT Y, KATO AC (1995) Quantitative comparison of the transient rescue effects of neurotrophic factors on axotomised motoneurons in vivo. *European Journal of Neuroscience* **7**, 108–115.

VERGE VM, MERLIO JP, GRONDIN J, ERNFORS P, PERSSON H, RIOPELLE RJ et al. (1992) Colocalisation of NGF binding sites, trk mRNA and low-affinity NGF receptor mRNA in primary sensory neurons: responses to injury and infusion of NGF. *Journal of Neuroscience* **12**, 4011–4022.

VERGE VMK, RICHARDSON PM, WIESENFELD-HALLIN Z, HÖKFELT T (1995) Differential influence of nerve growth factor on neuropeptide expression in vivo. A novel role in peptide suppression in adult sensory neurons. *Journal of Neuroscience* **15**, 2081–2096.

VERGE VMK, GRATTO KA, KARCHEWSKI LA, RICHARDSON PM (1996) Neurotrophins and nerve injury in the adult. *Philosophical Transactions of the Royal Society of London [Biological Sciences]* **351**, 423–430.

VERDI JM, GROVES AK, FARINAS I, JONES K, MARCHIONNI MA, REICHARDT LF et al. (1996) A reciprocal cell-cell interaction mediated by NT-3 and neuregulins controls the early survival and development of sympathetic neuroblasts. *Neuron* **16**, 515–527.

WAKISAKA S, TAKIKITA S, YOUN SH, KURISU K (1996) Partial coexistence of neuropeptide Y and calbindin D28k in the trigeminal ganglion following peripheral axotomy of the inferi alveolar nerve in the rat. *Brain Research* **707**, 228–234.

WANG W, SALVATERRA PM, LOERA S, CHIU AY (199 Brain derived neurotrophic factor spares choline acetyltran ferase mRNA following axotomy of motor neurons in viv *Journal of Neuroscience Research* **47**, 134–143.

WHITWORTH IH, TERENGHI G, GREEN CJ, BROWN R. STEVENS E, TOMLINSON DR (1995) Targeted delivery nerve growth factor via fibronectin conduits assists ner regeneration in control and diabetic rats. *European Journal Neuroscience* **7**, 2220–2225.

WHITWORTH IH, BROWN RA, DORE CJ, ANAND GREEN CJ, TERENGHI G (1996) Nerve growth fact enhances nerve regeneration through fibronectin grafts. *Journ of Hand Surgery* **21B**, 514–522.

WONG BJF, MATTOX (1991) Experimental nerve regeneratio *Otorhinolaryngology Clinics of North America* **24**, 739–752.

WONG J, OBLINGER MM (1991) NGF rescues substance expression but not neurofilaments or tubulin gene expression axotomised sensory neurons. *Journal of Neuroscience* **1** 543–552.

WOOLF CJ (1996) Phenotypic modification of primary senso neurons: the role of nerve growth factor in the production persistent pain. *Philosophical Transactions of the Royal Society London [Biological Sciences]* **351**, 441–448.

WRIGHT DE, SNIDER WD (1995) Neurotrophin recept mRNA expression defines distinct populations of neurons in dorsal root ganglia. *Journal of Comparative Neurology* **3** 329–338.

YAMAMORI T, FUKADA K, AEBERSOLD R, KORSCHIN S, FANN MJ, PATTERSON PH (1989) The choliner neuronal differentiation factor from heart cells is identical leukaemia inhibitory factor. *Science* **246**, 1412–1416.

YAN Q, ELLIOTT J, SNIDER WD (1992) Brain-deriv neurotrophic factor rescues spinal motor neurons from axoton induced cell death. *Nature* **360**, 753–755.

YAN Q, MATHESON C, LOPEZ OT (1995) In vivo neurotropi effects of GDNF on neonatal and adult facial motor neurc *Nature* **373**, 341–344.

YOU S, PETROV T, CHUNG PH, GORSON T (1997) T expression of the low affinity nerve growth factor receptor long-term denervated Schwann cells. *Glia* **20**, 87–100.

ZHANG L, SCHMIDT RE, YAN Q, SNIDER WD (1994) NC and NT-3 have differing effects on the growth of dorsal r axons in developing mammalian spinal cord. *Journal of Neu science* **14**, 5187–5201.

ZHANG X, JI RR, NILSSON S, VILLAR M, UBINK R, JU et al. (1995) Neuropeptide Y and galanin binding sites in rat a monkey lumbar dorsal root ganglia and spinal cord and effect peripheral axotomy. *European Journal of Neuroscience* 367–380.

ZHOU XF, RUSH RA (1995) Peripheral projections of rat prim sensory neurons immunoreactive for neurotrophin 3. *Journa Comparative Neurology* **363**, 69–77.

ZHOU XF, RUSH RA (1996) Endogenous brain-derived neu trophic factor is anterogradely transported in primary sens neurons. *Neuroscience* **74**, 945–951.

ZHOU XF, CHIE ET, DENG YS, RUSH RA (1997) Rat mat sympathetic neurones derive neurotrophin 3 from periph effector tissues. *European Journal of Neuroscience* **9**, 2753–27

J. Anat. (1999) 194, pp. 15–32 with 9 figures Printed in the United Kingdom

Review

Axon guidance of outgrowing corticospinal fibres in the rat

ELBERT A. J. JOOSTEN AND DOP P. R. BÄR

Department of Neurology, Laboratory of Experimental Neurology, Academic Hospital Utrecht, The Netherlands

(*Accepted 29 October 1998*)

ABSTRACT

This review is concerned with the development of the rat corticospinal tract (CST). The CST is a long descending central pathway, restricted to mammals, which is involved both in motor and sensory control. The rat CST is a very useful model in experimental research on the development of fibre systems in mammals because of its postnatal outgrowth throughout the spinal cord as well as its experimental accessibility. Hence mechanisms underlying axon outgrowth and subsequent target cell finding can be studied relatively easily. In this respect the corticospinal tract forms an important example and model system for the better understanding of central nervous system development in general.

Key words: Corticospinal tract; axon guidance; spinal cord; adhesion molecules; astroglia; dendrites; neurotropic.

INTRODUCTION

The rat pyramidal tract (PT) can be defined as a set of fibres passing through the medullary pyramids; its major component is the corticospinal tract (CST). The CST of the rat is a pathway which originates in layer V of the sensorimotor cortex. Its fibres descend through the internal capsule into the cerebral peduncles and extend caudally at the pial surface of the medulla oblongata (the medullary pyramids), and after decussation at the transition of the medulla to the spinal cord occupies the ventralmost part of the dorsal funiculus (Fig. 1) (DeMyer, 1967; Donatelle, 1977; Schreyer & Jones, 1982; Gribnau et al. 1986).

Although most corticospinal fibres are located within the ventralmost parts of the dorsal funiculus, minor CST components have been described: (1) a crossed component located in the dorsomedial parts of the lateral funiculus (Schreyer & Jones, 1982); (2) an ipsilateral uncrossed component located in the ventral funiculus (Vahlsing & Ferringa, 1980; Brosamle & Schwab, 1997).

In contrast to the lateral CST the major contralateral crossed component in the dorsal funiculus as well as the ipsilateral uncrossed component located in the ventral funiculus has been studied in great detail (Schreyer & Jones, 1982; Gribnau et al. 1986; Joosten et al. 1992). Using anterograde tract-tracing with horseradish peroxidase (HRP), labelled CST fibres initially extend as far as the ventral funiculus of lumbar spinal cord segments (Joosten et al. 1992). With the use of the HRP tracing technique it was shown that, after the first postnatal week, these lumbar projections disappear and the ipsilateral ventral CST projections were limited to lower cervical spinal cord segments (Joosten et al. 1992).

However, recent detailed studies with the very sensitive tracer biotin-dextran amine (BDA) unequivocally demonstrated the presence of labelled ipsilateral CST fibres into the ventral funiculus up to lumbar spinal cord levels also in adult rats (Herzog & Brosamle, 1997). The latter indicates how important the development of increasingly sensitive anterograde (or retrograde tracers) can be for a correct understanding of the development of central nervous system (CNS) projections.

Because the vast majority of the descending CST fibres (90–95%) is located within the dorsal funiculus this review on outgrowth and guidance of CST axons is focused on the major dorsal component. In order to

Correspondence to Dr Elbert A. J. Joosten, Room G02.314, Laboratory of Experimental Neurology, Academic Hospital Utrecht, Heidelberglaan 100, 3584 GA Utrecht,The Netherlands. Tel: +31 30 2507978; fax: +31 30 2542100; e-mail: EJoosten@neuro.azu.nl

The Second Galanin Receptor GalR2 Plays a Key Role in Neurite Outgrowth from Adult Sensory Neurons

Sally-Ann Mahoney,[1] Richard Hosking,[1] Sarah Farrant,[1] Fiona E. Holmes,[1] Arie S. Jacoby,[2] John Shine,[2] Tiina P. Iismaa,[2] Malcolm K. Scott,[3] Ralf Schmidt,[4] and David Wynick[1]

[1]University Research Centre for Neuroendocrinology, Bristol University, Bristol BS2 8HW, United Kingdom, [2]The Garvan Institute of Medical Research, Sydney, NSW 2010, Australia, [3]Johnson & Johnson Pharmaceutical Research Institute, Spring House, Pennsylvania 19477-0776, and [4]AstraZeneca R&D Montreal, Montreal, Quebec H4S 1Z9, Canada

Expression of the neuropeptide galanin is markedly upregulated within the adult dorsal root ganglion (DRG) after peripheral nerve injury. We demonstrated previously that the rate of peripheral nerve regeneration is reduced in galanin knock-out mice, with similar deficits observed in neurite outgrowth from cultured mutant DRG neurons. Here, we show that the addition of galanin peptide significantly enhanced neurite outgrowth from wild-type sensory neurons and fully rescued the observed deficits in mutant cultures. Furthermore, neurite outgrowth in wild-type cultures was reduced to levels observed in the mutants by the addition of the galanin antagonist M35 [galanin(1–13)bradykinin(2–9)]. Study of the first galanin receptor (GalR1) knock-out animals demonstrated no differences in neurite outgrowth compared with wild-type animals. Similarly, use of a GalR1-specific antagonist had no effect on neuritogenesis. In contrast, use of a GalR2-specific agonist had equipotent effects on neuritogenesis to galanin peptide, and inhibition of PKC reduced neurite outgrowth from wild-type sensory neurons to that observed in galanin knock-out cultures. These results demonstrate that adult sensory neurons are dependent, in part, on galanin for neurite extension and that this crucial physiological process is mediated by activation of the GalR2 receptor in a PKC-dependent manner.

Key words: galanin; GalR2; dorsal root ganglion; neuritogenesis; nerve injury; protein kinase C

Introduction

To date, the mechanisms and factors that regulate the regeneration of sensory neurons in the adult after peripheral nerve injury remain poorly understood. Damage to a peripheral nerve causes major changes within the cell bodies of the sensory neurons [dorsal root ganglion (DRG)], which are thought to promote regeneration by stimulating neurite outgrowth and enhancing survival of the damaged neuron. Furthermore, injury alters the retrograde flow of target-derived factors to the DRG. Examples of such phenomena include (1) the 120-fold upregulation in the levels of the neuropeptide galanin in the DRG after injury (Hokfelt et al., 1987) and (2) the marked increase in expression of the cytokines leukemia inhibitory factor (LIF) (Banner and Patterson, 1994; Dowsing et al., 1999) and interleukin 6 (IL-6) (Murphy et al., 1995) within Schwann cells at the site of injury and their retrograde transport to the DRG (Curtis et al., 1994). IL-6 and LIF have both been shown to promote axonal regeneration in the adult (Cheema et al., 1994; Hirota et al., 1996; Tham et al., 1997; Cafferty et al., 2001), and IL-6 knock-out mice have deficits in peripheral nerve regeneration after a crush injury to the sciatic nerve (Zhong et al., 1999). Recent data would indicate that LIF and IL-6 (acting through the gp130 coreceptor) may play a role in both injury-induced regeneration and minimizing pathological nociceptive responses by positively regulating the expression of galanin in the DRG (Corness et al., 1996; Thompson et al., 1998).

In the adult, galanin is expressed at low levels in <5% of DRG cells, which are predominantly the small peptidergic C-fiber neurons (Hokfelt et al., 1987). After nerve injury, there is a rapid and robust upregulation of both galanin mRNA and peptide, and expression of the peptide is now observed in 40–50% of all DRG neurons (Villar et al., 1989; Hokfelt et al., 1994). Studies indicate that galanin reduces transmission of sensory information in the spinal cord after nerve injury (Wiesenfeld-Hallin et al., 1989, 1992; Verge et al., 1993). In addition, rising levels of galanin in sensory neurons may also contribute to the initiation and maintenance of axonal regeneration in the injured neurons, leading to functional recovery and restoration of function while minimizing pathological nociceptive responses.

We showed previously that cultured DRG cells from animals homozygous for a targeted mutation in the galanin gene have a 35% reduction in the length of neurites and number of cells that extend neurites (Holmes et al., 2000). In this study, we used a combination of pharmacological and genetic tools to further elucidate the mechanisms by which galanin regulates neuritogenesis. Here, we show that galanin plays a neuritogenic role in adult sensory neurons and rescues the deficits in neurite outgrowth seen in mutant cultures. Furthermore, we demonstrate that its actions are mediated by the second galanin receptor (GalR2) in a PKC-dependent manner.

Materials and Methods

Animals

Galanin knock-out mice. Experiments were performed on 8-week-old female mice homozygous for a targeted mutation in the galanin gene.

Received July 19, 2002; revised Oct. 16, 2002; accepted Oct. 30, 2002.

This work was supported by the Medical Research Council, the Wellcome Trust, National Health and Medical Research Council (Australia), and a Human Science Program short-term fellowship.

Correspondence should be addressed to Prof. David Wynick, University Research Centre for Neuroendocrinology, Bristol University, Marlborough Street, Bristol BS2 8HW, UK. E-mail: d.wynick@bristol.ac.uk.

 0270-6474/03/230416-06$15.00/0

Age-matched wild-type littermates were used as controls in all experiments. Details of the strain and breeding history have been published previously (Wynick et al., 1998; Kerr et al., 2000). In brief, galanin knock-out mice were generated using the E14 cell line. A PGK-*Neo* cassette in reverse orientation was used to replace exons 1–5, and the mutation was bred to homozygosity and has remained inbred on the 129olaHsd strain. All animals were fed standard chow and water *ad libitum*. Animal care and procedures were performed within United Kingdom Home Office protocols and guidelines.

GalR1 knock-out mice. Experiments were performed on 8-week-old female mice that carry an insertional inactivating mutation within the first exon of the gene encoding the murine GalR1. Age-matched wild-type littermates were used as controls. In brief, GalR1 knock-out mice were generated using the W9.5 cell line and have remained inbred on the 129T2/SvEmsJ strain (Jacoby et al., 2002). All animals were fed standard chow and water *ad libitum*. Animal care and procedures were performed according to the Code of Practice of the Australian National Health and Medical Research Council.

DRG culture

Cultures were performed as described previously (Holmes et al., 2000). In brief, animals were killed by cervical dislocation, and DRGs from the lumbar, thoracic, and cervical regions were removed aseptically, trimmed of connective tissue and nerve roots, and pooled in DMEM–F12 medium. Ganglia were subjected to 0.25% collagenase P for 1 hr at 37°C, washed in PBS, and treated enzymatically with trypsin–EDTA for 10 min at 37°C. Ganglia were washed in medium containing trypsin inhibitor and then mechanically dissociated by trituration using a flame-narrowed Pasteur pipette. After centrifugation, cells were resuspended in DMEM–F12 medium supplemented with 5% horse serum, 1 mM glutamine, and 10 ng/ml gentamycin. To enhance the cultures for neurons and eliminate much of the cellular debris, cells were plated on six-well plates coated with 0.5 mg/ml polyornithine and maintained overnight at 37°C in a humidified incubator with 95% air–5% CO_2 (Patrone et al., 1999). Medium was removed and discarded. The neurons were removed from the surface by squirting with a jet of fresh medium. After centrifugation, cells were plated on 24-well plates treated with 0.5 mg/ml polyornithine and 5 μg/ml laminin and maintained for 8 hr at 37°C in a humidified incubator with 95% air–5% CO_2.

Treatments

Cells were cultured in DMEM–F12-supplemented medium as described above with or without the addition of the following chemicals: 1 nM M35 [galanin(1–13)bradykinin(2–9)] (Bachem UK, Essex, UK), 100 nM galanin peptide (Bachem UK), 10 μM bisindolylmaleimide I (BIM) (Calbiochem, La Jolla, CA), 10 nM RWJ-57408 [2,3-dihydro-2-(4-methyl-phenyl)-1,4-dithiepine-1,1,4,4-tetroxide] (Johnson & Johnson, Spring House, PA), and 100 nM AR-M961 ([Sar(1), D-Ala[12]Gal(1–16)-NH_2) or AR-M1896 [Gal(2–11)Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-NH_2] (AstraZeneca, Montreal, Quebec, Canada).

Data analysis

Cultures were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were visualized by phase-contrast microscopy. The percentage of cells bearing neurites and neurite length were both measured using NIH Image (Scion, Frederick, MD). Data are presented as mean ± SEM.

Results

Galanin signaling mediates neurite outgrowth

The addition of 100 nM galanin to wild-type adult DRG cells significantly increased the length of neurites (Fig. 1*B*), whereas the percentage of cells producing neurites was unchanged (Fig. 1*A*), suggesting that the number of cells capable of extending neurites was already at maximum under these culture conditions. Addition of 100 nM galanin peptide to mutant cultures fully rescued the deficits in the percentage of cells producing neurites and restored neurite length to wild-type levels.

We substantiated this putative neuritogenic role in the adult further by using the potent galanin antagonist M35 (Bartfai et al., 1992), which acts at all known galanin receptor subtypes (Wiesenfeld-Hallin et al., 1993). The addition of 1 nM M35 to wild-type cultures produced a significant 35% reduction in both the percentage of cells bearing neurites (Fig. 2*A*) and neurite length (Fig. 2*B*) to levels observed in mutant cultures. No effect was seen on either parameter in mutant cultures. This dose of M35 has been shown previously to have purely antagonistic effects (Wiesenfeld-Hallin et al., 1992; Ogren et al., 1993).

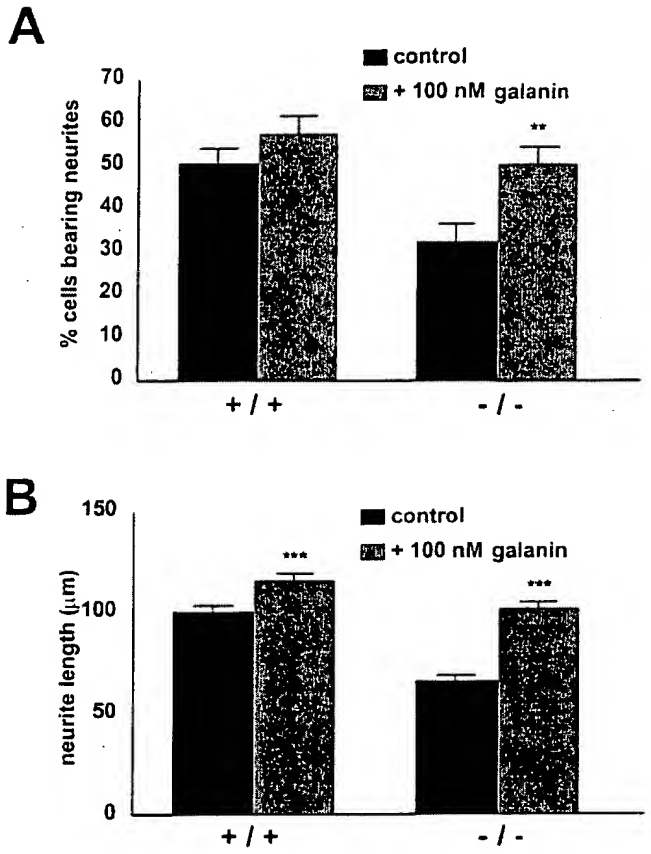


Figure 1. The percentage of cells bearing neurites (*A*) and length of neurite outgrowth (*B*) from dissociated DRG cultures isolated from wild-type and galanin knock-out animals in the presence and absence of 100 nM galanin peptide 8 hr after plating. Addition of 100 nM galanin to wild-type cultures significantly increased neurite length, whereas there was no significant difference in the percentage of cells producing neurites. However, addition of 100 nM galanin to galanin knock-out cultures significantly increased both the percentage of cells producing neurites and the length of neurites. Data are presented as percentage of cells bearing neurites or mean ± SEM length (*t* test; $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 5$).

The neuritogenic role of galanin is not mediated by GalR1

To determine whether the GalR1 receptor subtype is important in mediating the neuritogenic effects of galanin, we first used the small-molecule, nonpeptide GalR1-specific antagonist RWJ-57408 (Scott et al., 2000). The addition of 10 nM RWJ-57408 had no effect on the percentage of cells bearing neurites in either wild-type or mutant cultures (Fig. 3*A*). Similar results were seen with the addition of 1 nM RWJ-57408 (data not shown).

To study the role of GalR1 in mediating neuritogenesis further, we studied neurite outgrowth in GalR1 knock-out mouse dissociated DRG cultures (Jacoby et al., 2002). No differences were observed in the percentage of GalR1 mutant cells bearing neurites compared with those from wild-type controls (Fig. 3*B*).

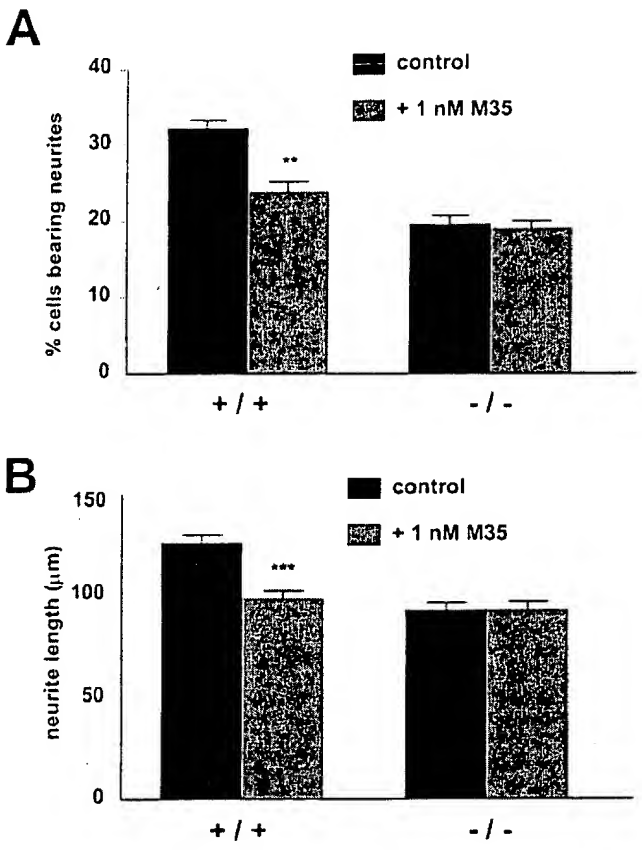


Figure 2. The percentage of cells bearing neurites (*A*) and length of neurite outgrowth (*B*) from dissociated DRG cultures isolated from wild-type and galanin knock-out animals in the presence and absence of 1 nM M35 8 hr after plating. In both cases, significant deficits were noted in the wild-type cultures, whereas no effect was seen in the knock-out cultures. Data are presented as percentage of cells bearing neurites or mean ± SEM length (*t* test; $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 5$).

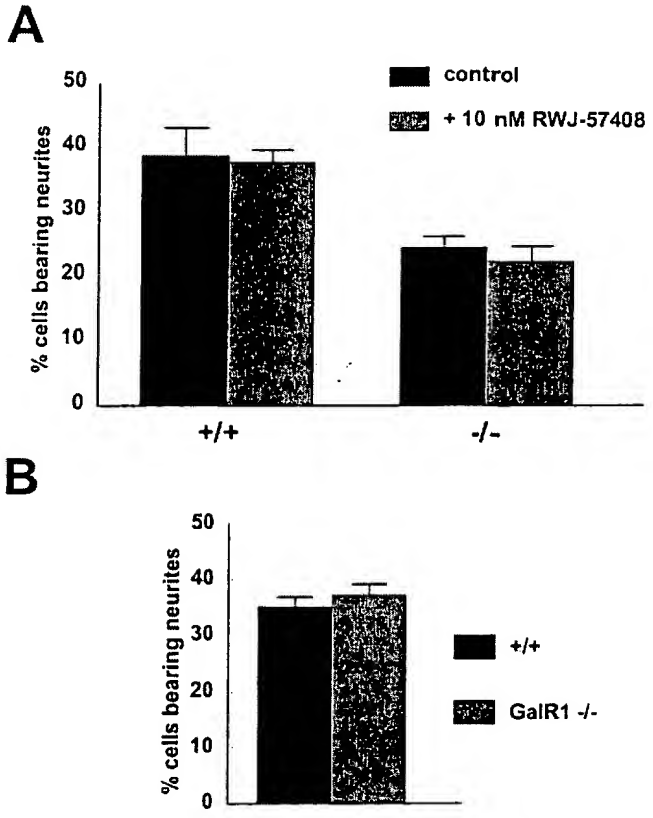


Figure 3. *A*, The percentage of cells bearing neurites from dissociated DRG cultures isolated from wild-type and galanin knock-out animals in the presence and absence of 10 nM RWJ-57408 8 hr after plating. *B*, The percentage of cells bearing neurites from dissociated DRG cultures isolated from wild-type and GalR1 knock-out animals 8 hr after plating. Data are presented as percentage of cells bearing neurites ± SEM ($n = 5$).

Furthermore, there was no significant difference in neurite length between cells from wild-type controls (140.8 ± 9 μm) and GalR1 mutant cells (161.6 ± 8 μm).

The neuritogenic role of galanin is mediated by GalR2 in a PKC-dependent manner

We next studied whether GalR2 might be responsible for transducing the neuritogenic actions of galanin by using the newly described peptide analog AR-M1896, a selective GalR2 agonist (Liu et al., 2001). In addition, we studied the actions of AR-M961, which has been shown to have agonistic actions at both GalR1 and GalR2 subtypes (Liu et al., 2001). The addition of 100 nM AR-M1896 or AR-M961 fully rescued the deficits in percentage outgrowth seen in the mutant cultures to wild-type levels (Fig. 4*A*) and appeared to be equipotent to galanin (Fig. 1*A*). Similarly, the addition of either AR-M961 or AR-M1896 to wild-type cultures increased both the percentage of cells bearing neurites and neurite length to the same extent as that observed with galanin peptide (Fig. 4*A*,*B*).

To investigate whether the neuritogenic role of galanin was PKC dependent, we used the PKC-specific inhibitor bisindolylmaleimide I (GF109203X) (Rivera-Bermudez et al., 2002). Addition of 10 μM BIM to wild-type cultures significantly reduced the percentage of cells bearing neurites to the levels observed in galanin knock-out cultures; similar results were seen using 1 μM BIM (data not shown). Furthermore, 10 μM BIM completely abolished the stimulatory neuritogenic actions of 100 nM AR-M1896 on both wild-type and mutant cultures (Fig. 4*C*).

Discussion

Damage to a peripheral nerve induces major and long-lasting changes in the expression of many secreted ligands and their receptors within the sensory neurons of the DRG. One of the most striking changes that occurs is the 120-fold increase in the expression of the neuropeptide galanin. We showed recently that galanin plays an important role in the survival of a subset of DRG neurons (Holmes et al., 2000), with a 2.8- and 2.6-fold increase in the number of apoptotic cells in the DRG of galanin knock-out mice at postnatal day 3 (P3) and P4, respectively, compared with wild-type controls. This wave of apoptosis at P3 is associated with a 13% decrease in total cell number within the DRG (Holmes et al., 2000). The role of galanin in cell survival is further substantiated by the finding that galanin is essential for the developmental survival of one-third of the cholinergic neurons of the basal forebrain (O'Meara et al., 2000).

The role played by galanin in the survival of this subset of DRG neurons seems to be preferentially biased toward the small peptidergic neurons, which are most likely to be nociceptors (Holmes et al., 2000). The loss of these small unmyelinated neurons may provide an explanation for the finding that galanin knock-out animals demonstrate a decrease in chronic neuropathic pain behavior after nerve injury (Kerr et al., 2000). Many of the animal models of peripheral nerve injury that induce neuropathic pain behavior are also associated with an upregulation

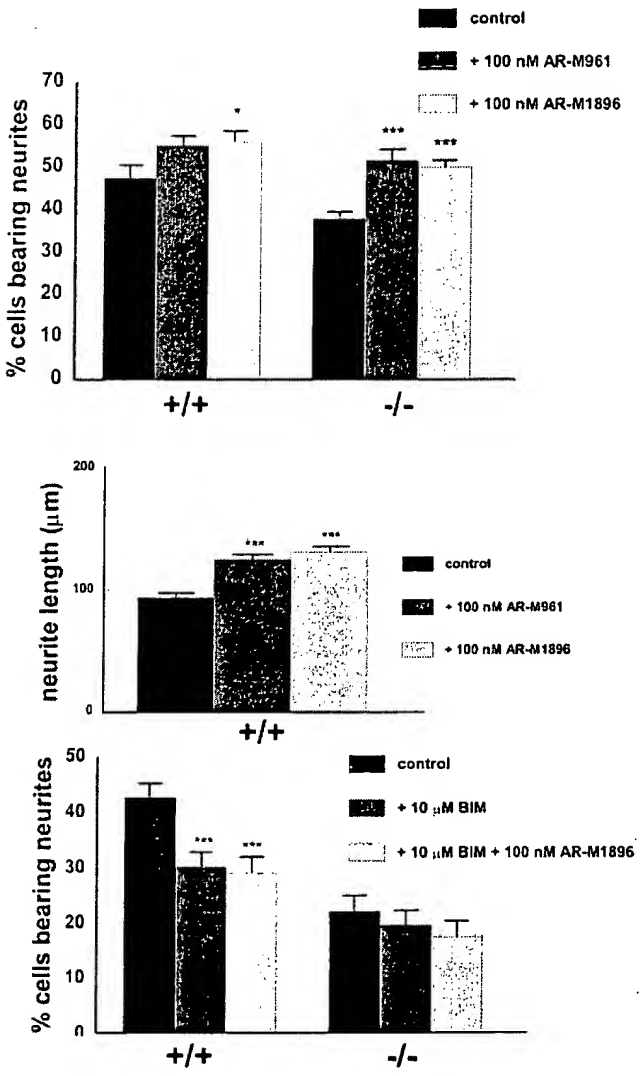


Figure 4. *A*, The percentage of cells bearing neurites from dissociated DRG cultures isolated from wild-type and galanin knock-out animals in the presence and absence of 100 nM AR-M961 or AR-M1896 8 hr after plating. The addition of either AR-M961 or AR-M1896 rescued the deficits in percentages of cells producing neurites seen in galanin knock-out cultures to near wild-type levels. Addition of AR-M1896 to wild-type cultures significantly increased the percentage of cells bearing neurites compared with controls. Although addition of AR-M961 increased the percentage, this was not significant. *B*, The length of neurite outgrowth from dissociated DRG cultures isolated from wild-type animals in the presence and absence of 100 nM AR-M961 or AR-M1896 8 hr after plating. Addition of either AR-M961 or AR-M1896 significantly increased neurite length. *C*, The percentage of cells bearing neurites from dissociated DRG cultures isolated from wild-type and galanin knock-out animals in the presence and absence of 10 μM BIM or 10 μM BIM plus AR-M1896. Significant deficits are seen in the number of cells producing neurites in wild-type cultures in the presence of 10 μM BIM, which was not rescued by the addition of 100 nM AR-M1896. Addition of 10 μM BIM had no effect on mutant cultures. Data are presented as percentage of cells bearing neurites or mean ± SEM length (*t* test; $*p < 0.05$; $***p < 0.001$; $n = 5$).

in galanin within the DRG neurons (Hokfelt et al., 1987; Villar et al., 1989; Nahin et al., 1994; Ma and Bisby, 1997). Furthermore, there appears to be a direct correlation between the extent and duration of pain behavior and the level of galanin upregulation (Ma and Bisby, 1997; Murphy et al., 1999).

The data presented here using galanin peptide or the potent galanin antagonist M35, which acts at all known galanin receptor subtypes (Wiesenfeld-Hallin et al., 1993), demonstrates that galanin is acting as a neuritogenic factor in the adult. Approximately one-third of neurite outgrowth in DRG cultures is dependent on the tonic release of galanin, implying that the developmental trophic–survival role is recapitulated in the adult after injury. At present, it is not possible to state whether there is a definite relationship between the early postnatal loss of a subset of small peptidergic neurons in the galanin knock-out animals and the reduced rate of neurite outgrowth in dispersed DRG cultures isolated from adult galanin knock-out animals. However, the finding that the deficits in neurite outgrowth that we identified in the adult galanin knock-outs are fully rescued by the addition of exogenous galanin would imply that the effects in the adult are independent of the developmental loss.

To date, three G-protein-coupled galanin receptor subtypes have been identified. GalR1 is expressed in the large-diameter neurons of the DRG, and GalR2 is expressed predominantly by the small- and medium-sized neurons. Only 5% of DRG neurons appear to express both receptor subtypes (Sten Shi et al., 1997). There have been contradictory reports as to whether GalR3 is expressed at all within the DRG. Studies using solution hybridization–RNase protection assays suggested that GalR3 is expressed at very low levels (Waters and Krause, 2000). However, other studies, again using solution hybridization–RNase protection assay (Smith et al., 1998), show no GalR3 present within the DRG; furthermore, no GalR3 has been detected in the DRG using riboprobe *in situ* hybridization (Mennicken et al., 2001). It is therefore unlikely that GalR3 plays a major role in neuritogenesis in the DRG.

Here, we demonstrate that addition of the small-molecule, nonpeptide GalR1-specific antagonist RWJ-57408 had no effect on neurite outgrowth in either wild-type or mutant cultures, suggesting that the role of galanin in neuritogenesis is not mediated via the GalR1 receptor subtype. This finding was substantiated by the finding that there was no difference in neurite outgrowth from GalR1 knock-out mice compared with wild-type controls. Furthermore, recent data using two separate *in vivo* models show that peripheral nerve regeneration is unaffected in GalR1 knock-out animals (Jacoby et al., 2002). These data therefore suggest that GalR1 does not mediate the neuritogenic or proregenerative effects of galanin in the DRG, implying that GalR2 is the predominant effector.

In this study, we used the galanin receptor agonists AR-M1896 and AR-M961 (Liu et al., 2001; Ma et al., 2001). AR-M1896 is a GalR2-specific agonist with an IC_{50} of 1.76 nM at rat GalR2 and 879 nM at human GalR1, whereas AR-M961 has been shown to have agonistic activity at both GalR2 and GalR1, with an IC_{50} of 1.74 nM and 0.403 nM, respectively (Liu et al., 2001). The data presented here demonstrate that both AR-M961 and AR-M1896 have positive effects on neuritogenesis and fully rescued deficits seen in the mutant cultures. Because our experiments using RWJ-57408 and GalR1 knock-out animals indicate that neurite outgrowth is not mediated via the GalR1 receptor, the agonistic action of AR-M961 on neurite outgrowth must be caused by activation of the GalR2 subtype, which is confirmed by the actions of the GalR2-specific agonist AR-M1896 (Liu et al., 2001).

The binding of galanin to GalR1 and GalR3 inhibits adenylyl cyclase (Habert-Ortoli et al., 1994; Smith et al., 1998; Wang et al., 1998), whereas binding to GalR2 stimulates principally phospholipase C activity (Fathi et al., 1997; Howard et al., 1997; Wang et al., 1997). Studies have shown that the G-protein-coupling profiles of GalR1 and GalR2 are distinct. GalR1 couples only to G_i, whereas GalR2 couples to G_i, G_o, and G_q (Wang et al., 1998). The G_i-mediated pathway is independent of PKC activity. In contrast, both the G_o- and G_q-mediated mitogen-activated protein kinase

signaling pathways are dependent on PKC activity (Hawes et al., 1996). Here, we demonstrate by use of the PKC-specific inhibitor BIM that the neuritogenic action of galanin is PKC dependent, which is consistent with activation of either the G_o- or G_q-mediated signaling pathways.

In summary, these results show that galanin is an important factor in neurite extension of adult sensory neurons and that this process is mediated by activation of GalR2 in a PKC-dependent manner. The role of GalR2 as a mediator of the proliferative effect of galanin is substantiated by previous findings in small-cell lung cancer cells (Wittau et al., 2000) and the pituitary (Wynick et al., 1993, 1998). Furthermore, previous studies have shown that the antiallodynic effect of galanin on neuropathic pain is mediated via GalR1 (Liu et al., 2001), whereas here we show that the neuritogenic role of galanin is mediated via GalR2. These results suggest that different receptor subtypes may be responsible for mediating the differing physiological roles of galanin in the adaptive response of the PNS to injury. Although few data are available on human galanin expression, it appears to have an expression pattern in the DRG similar to that of the rodent (Marti et al., 1987; Suburo et al., 1992). These findings have important implications for the potential therapeutic treatment of some peripheral sensory neuropathies by the use of selective GalR2 agonists.

References

Banner LR, Patterson PH (1994) Major changes in the expression of the mRNAs for cholinergic differentiation factor/leukemia inhibitory factor and its receptor after injury to adult peripheral nerves and ganglia. Proc Natl Acad Sci USA 91:7109–7113.

Bartfai T, Fisone G, Langel U (1992) Galanin and galanin antagonists: molecular and biochemical perspectives. Trends Pharmacol Sci 13:312–317.

Cafferty WB, Gardiner NJ, Gavazzi I, Powell J, McMahon SB, Heath JK, Munson J, Cohen J, Thompson SW (2001) Leukemia inhibitory factor determines the growth status of injured adult sensory neurons. J Neurosci 21:7161–7170.

Cheema SS, Richards L, Murphy M, Bartlett PF (1994) Leukemia inhibitory factor prevents the death of axotomised sensory neurons in the dorsal root ganglia of the neonatal rat. J Neurosci Res 37:213–218.

Corness J, Shi TJ, Xu ZQ, Brulet P, Hokfelt T (1996) Influence of leukemia inhibitory factor on galanin/GMAP and neuropeptide Y expression in mouse primary sensory neurons after axotomy. Exp Brain Res 112:79–88.

Curtis R, Scherer SS, Somogyi R, Adryan KM, Ip NY, Zhu Y, Lindsay RM, DiStefano PS (1994) Retrograde axonal transport of LIF is increased by peripheral nerve injury: correlation with increased LIF expression in distal nerve. Neuron 12:191–204.

Dowsing BJ, Morrison WA, Nicola NA, Starkey GP, Bucci T, Kilpatrick TJ (1999) Leukemia inhibitory factor is an autocrine survival factor for Schwann cells. J Neurochem 73:96–104.

Fathi Z, Cunningham AM, Iben LG, Battaglino PB, Ward SA, Nichol KA, Pine KA, Wang J, Goldstein ME, Iismaa TP, Zimanyi IA (1997) Cloning, pharmacological characterization and distribution of a novel galanin receptor. Brain Res Mol Brain Res 51:49–59.

Habert-Ortoli E, Amiranoff B, Loquet I, Laburthe M, Mayaux JF (1994) Molecular cloning of a functional human galanin receptor. Proc Natl Acad Sci USA 91:9780–9783.

Hawes BE, Luttrell LM, van Biesen T, Lefkowitz RJ (1996) Phosphatidylinositol 3-kinase is an early intermediate in the G beta gamma-mediated mitogen-activated protein kinase signaling pathway. J Biol Chem 271:12133–12136.

Hirota H, Kiyama H, Kishimoto T, Taga T (1996) Accelerated nerve regeneration in mice by upregulated expression of interleukin (IL) 6 and IL-6 receptor after trauma. J Exp Med 183:2627–2634.

Hokfelt T, Wiesenfeld-Hallin Z, Villar M, Melander T (1987) Increase of galanin-like immunoreactivity in rat dorsal root ganglion cells after peripheral axotomy. Neurosci Lett 83:217–220.

Hokfelt T, Zhang X, Wiesenfeld-Hallin Z (1994) Messenger plasticity in primary sensory neurons following axotomy and its functional implications. Trends Neurosci 17:22–30.

Holmes FE, Mahoney S, King VR, Bacon A, Kerr NC, Pachnis V, Curtis R, Priestley JV, Wynick D (2000) Targeted disruption of the galanin gene reduces the number of sensory neurons and their regenerative capacity. Proc Natl Acad Sci USA 97:11563–11568.

Howard AD, Tan C, Shiao LL, Palyha OC, McKee KK, Weinberg DH, Feighner SD, Cascieri MA, Smith RG, Van Der Ploeg LH, Sullivan KA (1997) Molecular cloning and characterization of a new receptor for galanin. FEBS Lett 405:285–290.

Jacoby AS, Holmes FE, Hort YJ, Shine J, Iismaa TP (2002) Phenotypic analysis of Galr1 knockout mice reveals a role for GALR1 galanin receptor in modulating seizure activity but not nerve regeneration. Lett Pept Sci 8:139–146.

Kerr BJ, Cafferty WB, Gupta YK, Bacon A, Wynick D, McMahon SB, Thompson SW (2000) Galanin knockout mice reveal nociceptive deficits following peripheral nerve injury. Eur J Neurosci 12:793–802.

Liu HX, Brumovsky P, Schmidt R, Brown W, Payza K, Hodzic L, Pou C, Godbout C, Hokfelt T (2001) Receptor subtype-specific pronociceptive and analgesic actions of galanin in the spinal cord: selective actions via GalR1 and GalR2 receptors. Proc Natl Acad Sci USA 98:9960–9964.

Ma W, Bisby MA (1997) Differential expression of galanin immunoreactivities in the primary sensory neurons following partial and complete sciatic nerve injuries. Neuroscience 79:1183–1195.

Ma X, Tong YG, Schmidt R, Brown W, Payza K, Hodzic L, Pou C, Godbout C, Hokfelt T, Xu ZQ (2001) Effects of galanin receptor agonists on locus coeruleus neurons. Brain Res 919:169–174.

Marti E, Gibson SJ, Polak JM, Facer P, Springall DR, Van Aswegen G, Aitchison M, Koltzenburg M (1987) Ontogeny of peptide- and amine-containing neurones in motor, sensory, and autonomic regions of rat and human spinal cord, dorsal root ganglia, and rat skin. J Comp Neurol 266:332–359.

Mennicken F, Hoffert C, Pelletier M, Ahmad S, Walker P, O'Donnell D (2001) Distinct distributions of GALR1, GALR2 and GALR3 mRNAs in the adult rat CNS and their modulation in pain models. Soc Neurosci Abstr 27:148.6.

Murphy PG, Grondin J, Altares M, Richardson PM (1995) Induction of interleukin-6 in axotomized sensory neurons. J Neurosci 15:5130–5138.

Murphy PG, Ramer MS, Borthwick L, Gauldie J, Richardson PM, Bisby MA (1999) Endogenous interleukin-6 contributes to hypersensitivity to cutaneous stimuli and changes in neuropeptides associated with chronic nerve constriction in mice. Eur J Neurosci 11:2243–2253.

Nahin RL, Ren K, De Leon M, Ruda M (1994) Primary sensory neurons exhibit altered gene expression in a rat model of neuropathic pain. Pain 58:95–108.

Ogren SO, Pramanik A, Land T, Langel U (1993) Differential effects of the putative galanin receptor antagonists M15 and M35 on striatal acetylcholine release. Eur J Pharmacol 242:59–64.

O'Meara G, Coumis U, Ma SY, Kehr J, Mahoney S, Bacon A, Allen SJ, Holmes F, Kahl U, Wang FH, Kearns IR, Ove-Ogren S, Dawbarn D, Mufson EJ, Davies C, Dawson G, Wynick D (2000) Galanin regulates the postnatal survival of a subset of basal forebrain cholinergic neurons. Proc Natl Acad Sci USA 97:11569–11574.

Patrone C, Andersson S, Korhonen L, Lindholm D (1999) Estrogen receptor-dependent regulation of sensory neuron survival in developing dorsal root ganglion. Proc Natl Acad Sci USA 96:10905–10910.

Rivera-Bermudez MA, Bertics PJ, Albrecht RM, Mosavin R, Mellon WS (2002) 1,25-Dihydroxyvitamin D_3 selectively translocates PKCalpha to nuclei in ROS 17/2.8 cells. Mol Cell Endocrinol 188:227–239.

Scott MK, Ross TM, Lee DH, Wang HY, Shank RP, Wild KD, Davis CB, Crooke JJ, Potocki AC, Reitz AB (2000) 2,3-Dihydro-dithiin and -dithiepine-1,1,4,4-tetroxides: small molecule non-peptide antagonists of the human galanin hGAL-1 receptor. Bioorg Med Chem 8:1383–1391.

Smith KE, Walker MW, Artymyshyn R, Bard J, Borowsky B, Tamm JA, Yao WJ, Vaysse PJ, Branchek TA, Gerald C, Jones KA (1998) Cloned human and rat galanin GALR3 receptors: pharmacology and activation of G-protein inwardly rectifying K^+ channels. J Biol Chem 273:23321–23326.

Sten Shi TJ, Zhang X, Holmberg K, Xu ZQ, Hokfelt T (1997) Expression and regulation of galanin-R2 receptors in rat primary sensory neurons: effect of axotomy and inflammation. Neurosci Lett 237:57–60.

Suburo AM, Gu XH, Moscoso G, Ross A, Terenghi G, Polak JM (1992) Developmental pattern and distribution of nerve growth factor low-affinity receptor immunoreactivity in human spinal cord and dorsal root ganglia: comparison with synaptophysin, neurofilament and neuropeptide immunoreactivities. Neuroscience 50:467–482.

Tham S, Dowsing B, Finkelstein D, Donato R, Cheema SS, Bartlett PF, Morrison WA (1997) Leukemia inhibitory factor enhances the regeneration of transected rat sciatic nerve and the function of reinnervated muscle. J Neurosci Res 47:208–215.

Thompson SW, Priestley JV, Southall A (1998) gp130 cytokines, leukemia inhibitory factor and interleukin-6, induce neuropeptide expression in intact adult rat sensory neurons in vivo: time-course, specificity and comparison with sciatic nerve axotomy. Neuroscience 84:1247–1255.

Verge VM, Xu XJ, Langel U, Hokfelt T, Wiesenfeld-Hallin Z, Bartfai T (1993) Evidence for endogenous inhibition of autotomy by galanin in the rat after sciatic nerve section: demonstrated by chronic intrathecal infusion of a high affinity galanin receptor antagonist. Neurosci Lett 149:193–197.

Villar MJ, Cortes R, Theodorsson E, Wiesenfeld-Hallin Z, Schalling M, Fahrenkrug J, Emson PC, Hokfelt T (1989) Neuropeptide expression in rat dorsal root ganglion cells and spinal cord after peripheral nerve injury with special reference to galanin. Neuroscience 33:587–604.

Wang S, He C, Hashemi T, Bayne M (1997) Cloning and expressional characterization of a novel galanin receptor: identification of different pharmacophores within galanin for the three galanin receptor subtypes. J Biol Chem 272:31949–31952.

Wang S, Hashemi T, Fried S, Clemmons AL, Hawes BE (1998) Differential intracellular signaling of the GalR1 and GalR2 galanin receptor subtypes. Biochemistry 37:6711–6717.

Waters SM, Krause JE (2000) Distribution of galanin-1, -2 and -3 receptor messenger RNAs in central and peripheral rat tissues. Neuroscience 95:265–271.

Wiesenfeld-Hallin Z, Xu XJ, Villar MJ, Hokfelt T (1989) The effect of intrathecal galanin on the flexor reflex in rat: increased depression after sciatic nerve section. Neurosci Lett 105:149–154.

Wiesenfeld-Hallin Z, Xu XJ, Langel U, Bedecs K, Hokfelt T, Bartfai T (1992) Galanin-mediated control of pain: enhanced role after nerve injury. Proc Natl Acad Sci USA 89:3334–3337.

Wiesenfeld-Hallin Z, Xu XJ, Hao JX, Hokfelt T (1993) The behavioural effects of intrathecal galanin on tests of thermal and mechanical nociception in the rat. Acta Physiol Scand 147:457–458.

Wittau N, Grosse R, Kalkbrenner F, Gohla A, Schultz G, Gudermann T (2000) The galanin receptor type 2 initiates multiple signaling pathways in small cell lung cancer cells by coupling to G_q, G_i and G_{12} proteins. Oncogene 19:4199–4209.

Wynick D, Hammond PJ, Akinsanya KO, Bloom SR (1993) Galanin regulates basal and oestrogen-stimulated lactotroph function. Nature 364:529–532.

Wynick D, Small CJ, Bacon A, Holmes FE, Norman M, Ormandy CJ, Kilic E, Kerr NC, Ghatei M, Talamantes F, Bloom SR, Pachnis V (1998) Galanin regulates prolactin release and lactotroph proliferation. Proc Natl Acad Sci USA 95:12671–12676.

Zhong J, Dietzel ID, Wahle P, Kopf M, Heumann R (1999) Sensory impairments and delayed regeneration of sensory axons in interleukin-6-deficient mice. J Neurosci 19:4305–4313.

NEUROPEPTIDES

Neuropeptides (1995) 29, 137–143

Metabolism of Galanin and Galanin (1–16) in Isolated Cerebrospinal Fluid and Spinal Cord Membranes from Rat

K. BEDECS, Ü. LANGEL and T. BARTFAI

Department of Neurochemistry and Neurotoxicology, Stockholm University, Frescati, S-10691 Stockholm, Sweden

Abstract—The occurrence of galanin (GAL) in the spinal cord and reports suggesting that it acts as an endogenous inhibitory spinal modulator in sensory/noxious transmission, have focused interest on its metabolism in the spinal cord. The metabolic half-lives and degradation patterns of GAL(1–29) and the high affinity N-terminal fragment GAL(1–16), were determined in isolated cerebrospinal fluid (CSF) from rats, and analysed by reverse phase HPLC. The half-lives for GAL(1–29) and GAL(1–16) in isolated rat CSF at 37°C were 120 min and 60 min, respectively. The first degradation products which we could isolate and identify of GAL(1–16) were: GAL(3–16) and GAL(3–12) and for GAL(1–29): GAL(1–5) and GAL(1–4), all without affinity to spinal galanin receptors. Degradation studies of GAL(1–29) and GAL(1–16) in a spinal cord membrane preparation, in absence or presence of different protease inhibitors: E-64, pepstatin A, 3,4-DCI, bestatin, phosphoramidon, kelatorphan and thiorphan, or metal chelators: EDTA, EGTA and *o*-phenanthrolin, suggest that a phosphoramidon sensitive zinc-metalloprotease is mainly responsible for the degradation of GAL(1–29) and GAL(1–16), since both *o*-phenanthrolin (0.3 mM) and phosphoramidon (920 μM) substantially prolong their half-lives.

Introduction

Galanin (GAL)[1] a 29/30 amino acid long C-terminally amidated neuropeptide is widely distributed and exerts a variety of biological actions in the peripheral and central nervous system. In the rat spinal cord GAL is present in primary sensory neurones (DRG), local dorsal horn cells and motor neurones.[2,3] GAL is of particular interest as an endogenous inhibitory spinal modulator in sensory/noxious processing and transmission. Intrathecally (i.t.) applied GAL has a concentration dependent biphasic facilitatory and depressive effect on the spinal flexor reflex (used as a model for nociceptive and sensory transmission)[4] and dose dependently inhibits the facilitatory effect 'wind-

Date received 27 October 1994
Date accepted 26 February 1995

Correspondence to Tamas Bartfai, Department of Neurochemistry and Neurotoxicology, Stockholm University, Arrhenius Laboratories for Natural Sciences, S-10691 Stockholm, Sweden

up' of conditioning stimulation (CS) of C-afferents in skin and muscle nerves.[5] I.t. GAL also potentiates the spinal anti-nociceptive effect of morphine and a selective CCK-B antagonist at a dose which by itself does not cause analgesia.[6,7]

The occurrence of GAL in the central nervous system and the possibility that it may act as an endogenous inhibitory modulator have focused interest on its metabolism. Fast enzymatic inactivation is probably of importance in terminating the actions of the synaptically released peptide neurotransmitters. Pharmacological use of peptide receptor ligands assumes that we can adequately protect these from peptidolysis either by finding non-peptide ligands (e.g. morphine) or by synthesizing protected peptide type ligands. If the pharmacological effect of the peptide receptor agonist is desirable, inhibitors to specific degrading enzymes may also be useful. The first step toward these goals is to establish the degradation pattern for the peptide in question.

Earlier studies have shown that GAL is degraded rapidly when incubated with gastric smooth muscle membranes, although the metabolic products or half-life in this preparation were not determined.[8] Degradation of GAL(1–29) and GAL(1–16) (a high-affinity agonist) in a hypothalamic crude membrane preparation showed that the metabolic half-lives were 100 min and 28 min, respectively. The proposed peptidolytic cleavage sites in GAL(1–16) were between Trp^2-Thr^3, Thr^3-Leu^4, and Leu^4-Asn^5, yielding fragments GAL(3–16), GAL(4–16) and GAL(5–16), all without measurable affinity to the GAL receptor.[9]

During an intrathecal administration, GAL is injected into the cerebrospinal fluid (CSF), permeates the pia mater and diffuses to the superficial layers (laminae I and II) of the spinal cord, where spinal GAL receptors are expressed. The metabolism of endogenously occurring GAL(1–29) and the N-terminal biologically active GAL fragment GAL(1–16) has not yet been studied in the spinal cord or CSF. Identification of the fissile bonds in GAL(1–29) and GAL(1–16) may assist the synthesis of peptidolytically more stable, long acting analogues which could be used in pain treatment.

The present study shows the metabolism of GAL(1–29) and the GAL(1–16) in isolated rat CSF and a P_2-membrane preparation from the lumbar dorsal spinal cord.

Materials and methods

Materials

Rat GAL(1–29) and GAL(1–16) were synthesized by the *t*-Boc method, purified on a LKB HPLC apparatus (SYSTEM PREP 50) using Polygosil 60–7 C_{18} reverse phase column and characterized by plasma desorption mass spectrometry, model Bioion 20, Applied Biosystems, as described by Langel et al 1992.[10] GAL(1–29) was synthesized with a C-terminal amide corresponding to the endogenous peptide while GAL(1–16) was synthesized as a free carboxylic acid. The protease inhibitors E-64 (N-[N-(L-3-Trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine), 3, 4-DCI (3, 4-Dichloroisocoumarin), pepstatin A, phosphoramidon, bestatin and thiorphan were from Boehringer Mannheim. Kelatorphan was a generous gift from Prof. B. Roques (Paris). All other reagents were from Sigma (St Louis, MO, USA).

Isolation of CSF

Adult male rats (Sprague-Dawley, 350–400 g) were anaesthetised with chloral hydrate 300 mg/kg body weight (i.p.) and CSF was collected from the Cisternae cerebello medullaris through a suboccipital puncture and immediately put on ice. To remove contaminating blood cells the CSF was centrifuged for 2 min at 11 000 × g.

Control experiments were performed, with serum from blood collected through a heart puncture, and showed no degradation of either GAL(1–29) or GAL(1–16).

Preparation of membranes from lumbar dorsal spinal cord

Adult male rats (Sprague-Dawley 180–200 g) were decapitated, the lumbar spinal cord rapidly dissected and divided into dorsal and ventral parts. The tissue (10% w/v) was homogenized on ice with a tight fitting Teflon-glass homogenizer (10 strokes at 695 rpm) in 0.32 M sucrose buffered with 5 mM Hepes (pH 7.4). The homogenate was diluted 10-fold with 0.32 M sucrose buffered with 5 mM Hepes

(pH 7.4) and centrifuged at 1000 × g for 10 min. The supernatant was further centrifuged at 10 000 × g for 45 min and the pellet resuspended at a concentration of 1 mg protein/ml in Hepes (5 mM)-buffered Krebs-Ringer solution (Hepes-KR), containing 137 mM NaCl, 2.68 mM KCl, 1.8 mM $CaCl_2$, 2.05 mM $MgCl_2$ and 1 g/l glucose, pH 7.4, aliquoted and stored at −80°C.

Degradation of GAL(1–29) and GAL(1–16) in CSF or lumbar dorsal spinal cord membranes

To determine the rate of degradation of and to identify the proteolytic products, GAL(1–29) and GAL(1–16) at a concentration of 1 and 0.5 μg/μl, respectively, were incubated with isolated rat CSF or P_2-membranes from the lumbar dorsal spinal cord (0.6 mg protein/ml, GAL(1–29) and GAL(1–16): 30 μM) at 37°C, 0–210 min. The degradation was stopped by precipitation of proteins with perchloric acid (final concentration 2% (v/v)), and centrifuged for 2 min at 11 000 × g. The resulting supernatant was analysed by HPLC on C18 reverse phase analytical column (Nucleosil 120–3, 100 × 4 mm ID), eluting with a 16–56% acetonitrile/water (0.1% TFA v/v), gradient for 50 min, flowrate 0.8 ml/min.

To characterize the degradation products, the different peaks were collected, lyophilised and Phenylisothiocyanate (PITC) amino acid analysis was performed.

First order rate constants of the disappearance of GAL(1–29) and GAL(1–16) were calculated according to equation 1.

$$S_t = S_o\, e^{-kt} \qquad \text{(eq. 1)}$$

where S_t = area of non-degraded peptide peak, at time moment t; S_o = area of initial peptide peak at t = 0; k = first order rate constant of the degradation of the peptides.

Results

To determine the half-lives and degradation pattern of GAL(1–29) and GAL(1–16), degradation studies were performed in freshly isolated CSF from rats. The rate of disappearance of GAL(1–29) and GAL(1–16), and the appearance of degradation products were followed by HPLC-analysis. Those degradation products which appeared first were collected and further characterized by PITC amino acid analysis. In the isolated CSF preparation the metabolic half-lives for GAL(1–29) and GAL(1–16) were 120 ± 60 min (n = 6) and 60 ± 30 min (n = 6), respectively at 37°C. The first independently appearing degradation products for GAL(1–16) were GAL(3–16) and GAL(3–12) and for GAL(1–29) were GAL(1–5) and GAL(1–4). None of these peptide fragments could displace ^{125}I-GAL(1–29) binding from spinal cord membranes (Fig. 1).

To characterize the protease class which is mainly responsible for the degradation of GAL(1–16) and GAL(1–29) we used a lumbar dorsal spinal cord P_2-membrane preparation, because of the poor reproducibility of half-lives of GAL(1–29) and GAL(1–16) in isolated CSF. Degradation studies, in absence or presence of different protease inhibitors were performed.

In Figure 2 the half-lives of GAL(1–16) and GAL(1–29) in presence of the serine, cysteine and aspartate protease inhibitors 3,4-DCI (0.1 mM), E-64 (1.4 mM) and pepstatin (20 μM), respectively are listed. As shown, neither one of these inhibitors is able to prolong the half-lives of GAL(1–16) or GAL(1–29). However, the metal chelators EDTA, EGTA and *o*-phenanthrolin (1,10-phenanthrolin) significantly prolonged the half-lives of GAL(1–16) and GAL(1–29) in spinal cord membranes. Whereas the Zn^{2+} chelator *o*-phenanthrolin at 0.2 mM prolonged the half-lives of GAL(1–16) and GAL(1–29) three times, EDTA and EGTA (10 mM) could prolong the half-life of GAL(1–29) 6 times, but the half-life of GAL(1–16) only 3 times. The results presented in Figure 2 clearly indicate that GAL(1–16) and GAL(1–29) are sensitive to metalloprotease(s), probably Zn-metalloprotease(s).

To further characterize which metalloproteases are involved in the degradation of GAL(1–16) and GAL(1–29), their half-lives were measured in the presence of inhibitors to some well characterized metalloproteases, known to be present in the CSF and spinal cord. In Figure 3, the half-lives of GAL(1–16) and GAL(1–29) are shown in the presence of the peptidase inhibitors: bestatin (aminopeptidase N/M (APN/M)), phosphoramidon (neutral endopeptidase EC. 3.4.24.11 (NEP)), thior-

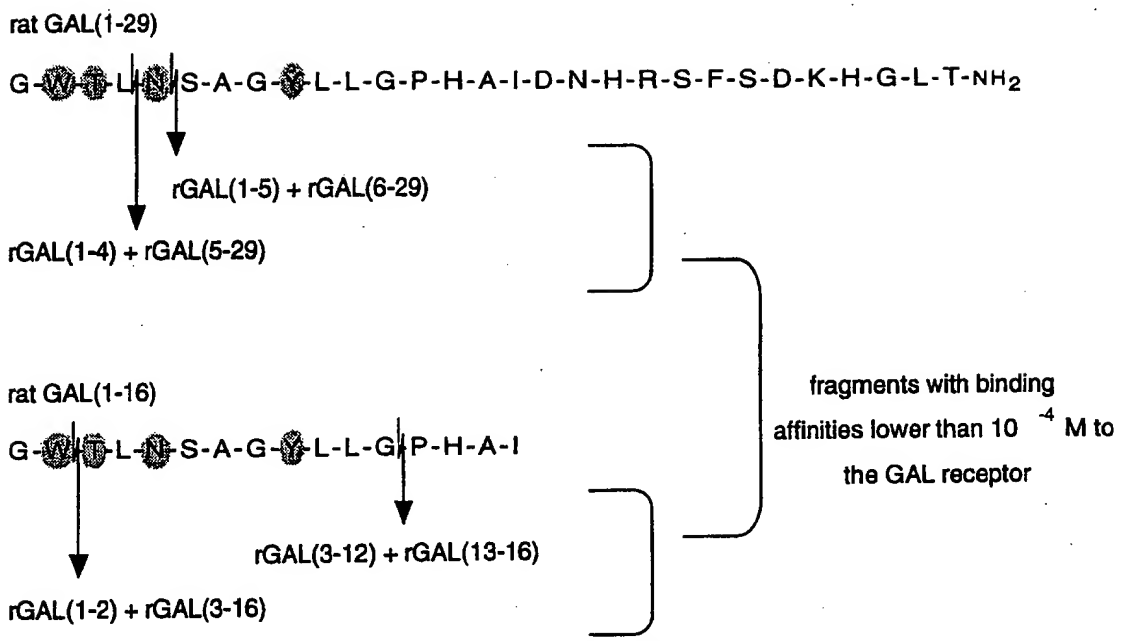


Fig. 1 Degradation patterns of GAL(1–29) and GAL(1–16) in isolated rat CSF. Encircled letters indicate main pharmacophores in binding to the GAL receptor

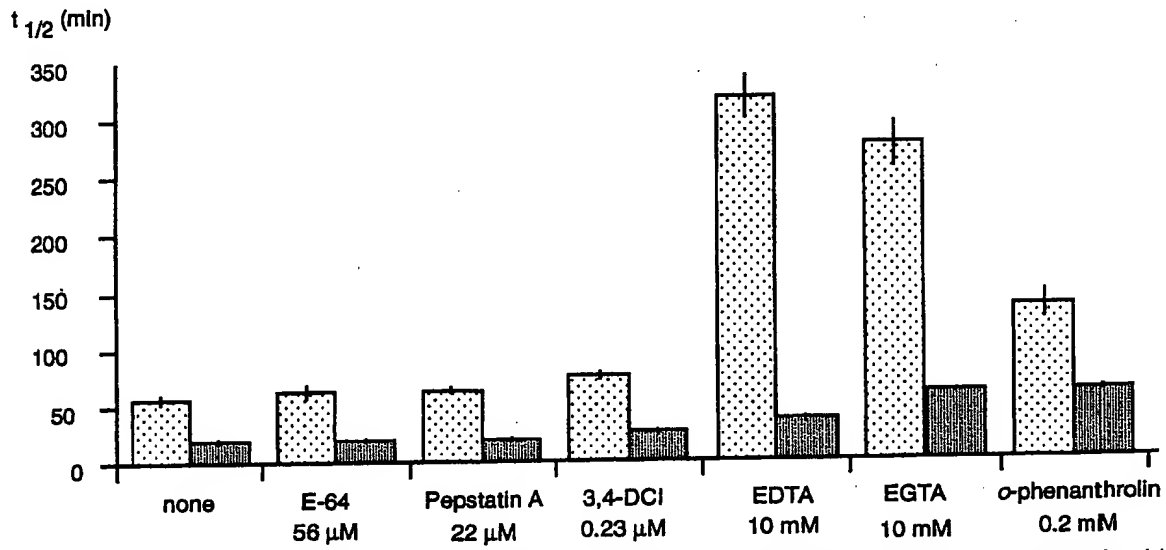


Fig. 2 The half-lives of GAL(1–16) (dark columns) and GAL(1–29) (light columns) [30 µM] incubated in a crude mitochondrial membrane preparation (P_2) [0.6 mg/ml] from LDSC, at 37°C, in presence of the protease inhibitors 3,4-DCI (serine), E-64 (cysteine), pepstatin A (aspartate) and metal-chelating agents *o*-phenanthrolin, EDTA and EGTA. The rate of peptide degradation was followed on HPLC and the half-lives were calculated according to $S_t = S_o e^{-kt}$, where S_t = area of non-degraded peptide peak at time moment t; S_o = area of initial peptide peak at t = O; k = first order rate constant of the degradation of the peptides.

phan (NEP), kelatorphan (mixed APN/M-NEP inhibitor), and a mixture of these. Of these metalloprotease inhibitors, only the endopeptidase inhibitor phosphoramidon, could significantly inhibit the degradation of GAL(1–16) and GAL(1–29).

The degradation of both GAL(1–29) and GAL(1–16) was faster when incubated with mem-

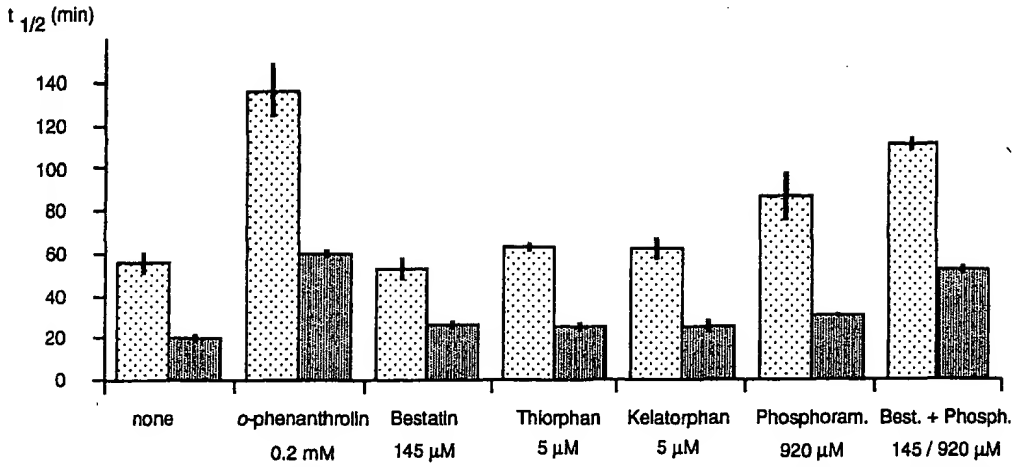


Fig. 3 The half-lives of GAL(1–16) (dark columns) and GAL(1–29) (light columns) [30 µM] incubated in a crude mitochondrial membrane preparation (P_2) [0.6 mg/ml] from LDSC, at 37°C, in presence of the protease inhibitors bestatin (APN/M), phosphoramidon (NEP), thiorphan (NEP), kelatorphan (mixed APN/M-NEP inhibitor), and a mixture of these. For calculations see legend under Figure 2.

branes from spinal cord than when incubated with isolated CSF, which can be explained by the membrane concentration used, in this case 0.6 mg protein/ml. To determine whether or not the inhibition of degradation of GAL(1–16) and GAL(1–29) by *o*-phenanthrolin was concentration-dependent, degradation of GAL(1–16) and GAL(1–29) in spinal cord membranes in the presence of increasing concentrations of *o*-phenanthrolin was studied (Fig. 4). The degradation of both GAL(1–16) and GAL(1–29) was inhibited in a concentration-dependent manner by *o*-phenanthrolin. At 0.4 mM (the highest inhibitor concentration used) the half-life of GAL(1–16) was prolonged 12-fold (from 22 to 270 min) and for GAL(1–29) the half-life was prolonged only 5-fold (from 70 to 398 min). In a preliminary study of the degradation of GAL(1–16) and GAL(1–29) in isolated human CSF (frozen) the half-lives for GAL(1–29) and GAL(1–16) were $\gg$ 180 min, probably due to inactivation of degrading activities during storage of the samples.

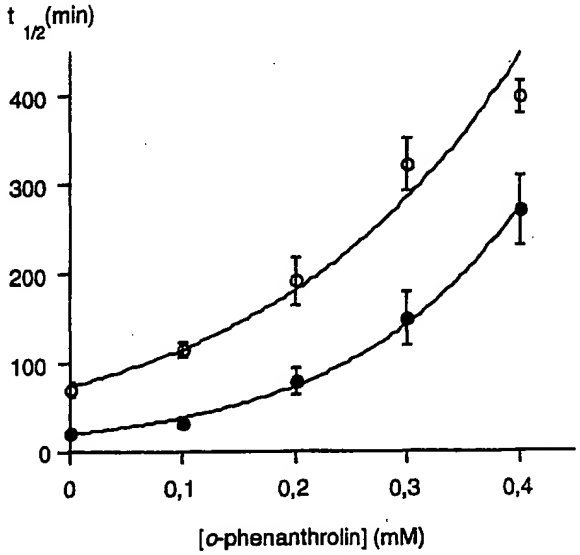


Fig. 4 Metabolic half-lives of GAL(1–16) (filled circles) and GAL(1–29) (open circles) incubated in P_2 membranes from lumbar dorsal spinal cord, 37°C, in presence of increasing concentrations of *o*-phenanthrolin.

Discussion

The present study shows that GAL(1–16) and GAL(1–29) are enzymatically degraded in isolated CSF from rat. Although GAL(1–16) has not been shown to occur endogenously, information about its metabolism is important from a pharmacological-therapeutic point of view, since it acts as a high affinity full agonist.[11] Studies on the structural requirements of the biological activity of GAL(1–29) in the spinal cord have shown that the N-terminal fragment GAL(1–16) is an equipotent agonist at the spinal GAL receptor, with a K_D value of 3 nM, but with slightly superior pharmaco-kinetic properties e.g. penetration and diffusion constants compared to GAL(1–29).[11]

The half-lives for GAL(1–16) and GAL(1–29) in

CSF were 60 ± 30 min and 120 ± 60 min, respectively, showing that GAL is a surprisingly stable peptide. The half-lives for GAL(1–16) and GAL(1–29) in the spinal cord membrane preparation were 20 ± 2 and 56 ± 5 min, respectively. An earlier study carried out in a hypothalamic membrane preparation, showed that the half-lives for GAL(1–16) and GAL(1–29) were 28 and 100 min, respectively.[9]

The higher metabolic stability of GAL(1–29) as compared to GAL(1–16), can be explained by the proposed horse-shoe like conformation of GAL(1–29) based on fluorescence energy transfer data, suggesting that the C-terminal part of the molecule can sterically protect the N-terminal and render it less susceptible for proteolytic attack.[12,13]

The first appearing degradation products for GAL(1–16) were GAL(3–16) and GAL(3–12) and for GAL(1–29) were GAL(1–4) and GAL(1–5) as determined by PITC amino acid analysis of the collected HPLC peaks/fractions. To address the question whether the formation of GAL(3–12) was secondary to a previous formation of GAL(3–16) from GAL(1–16), synthetic GAL(3–16) was incubated with isolated CSF for 90 min. No formation of GAL(3–12) was observed, indicating that GAL(3–16) and GAL(3–12) are independent degradation products of GAL(1–16). To yield the fragment GAL(3–12), GAL(1–16) is cleaved between Trp^2-Thr^3 and Pro^{12}-Gly^{13}, where the latter represents an unusual cleavage site. The degradation patterns of GAL(1–16) and GAL(1–29) in CSF showed that the cleavage sites are located mainly in the N-terminal part of the peptides. Cleavage in the N-terminal part of GAL(1–16) and GAL(1–29) represents a biologically significant degradation, as this results in fragments which no longer bind to spinal GAL receptors with high affinity, and are thus in a galaninergic perspective, inactive fragments. In order to characterize which protease class is mainly responsible for the degradation of GAL(1–16) and GAL(1–29) we changed the model system from isolated CSF to a lumbar dorsal spinal cord P_2-membrane preparation.

The degradation profile (i.e. time dependency and amount of individual degradation products) of GAL(1–16) and GAL(1–29) in spinal cord membranes were similar to that in CSF, suggesting that a similar set of peptidases is acting in the CSF and in the spinal cord membranes. The inability of the serine (3.4-DCI), cysteine (E-64) and aspartate (pepstatin) protease inhibitors to prolong the half-lives of GAL(1–16) and GAL(1–29) and the potent inhibition by *o*-phenanthrolin, EDTA and EGTA suggest that metalloproteases are active in the metabolism of GAL(1–16) and GAL(1–29) (Fig. 2). An interesting finding was that GAL(1–16) and GAL(1–29) were differently inhibited by the metal chelators *o*-phenanthrolin, EDTA and EGTA. *o*-phenanthrolin prolonged the half-lives of GAL(1–16) and GAL(1–29) three times, whereas EDTA and EGTA prolonged the half-life of GAL(1–29) six times, but the half-life of GAL(1–16) only three times. This may indicate that metalloproteases other than Zn-metalloproteases are also active in the degradation of the C-terminal part of GAL: GAL(17–29), whereas the N-terminal part is degraded mainly by Zn-metalloproteases.

In the subsequent investigation of which metalloproteases degrade GAL(1–16) and GAL(1–29), we selected inhibitors specific for NEP (thiorphan), APN/M (bestatin), mixed NEP and APN/M inhibitor (kelatorphan) and the less specific metallo-endopeptidase inhibitor phosphoramidon, which is also a potent NEP inhibitor. These studies suggest that a metallo-endopeptidase(s), other than NEP degrades GAL(1–16) and GAL(1–29) in the spinal cord membranes, since phosphoramidon but neither thiorphan nor kelatorphan significantly prolonged their half-lives. Phosphoramidon (920 μM) prolonged the half-lives of both GAL(1–16) and GAL(1–29) to the same extent(1.5-fold), indicating that the protease inhibited by phosphoramidon, is active in the GAL(1–16) part of GAL(1–29). The identification of the first appearing degradation products of GAL(1–16) i.e. GAL(3–16) and GAL(3–12) and of GAL(1–29), GAL(1–4) and GAL(1–5) in isolated CSF may suggest the involvement of (N-terminally acting) amino- and/or dipeptidyl amino peptidases. This is consistent with a study in a hypothalamic membrane preparation, where the degradation of GAL(1–29) and GAL(1–16) was shifted from the N-terminal portion to the C-terminal portion in the presence of EDTA, suggesting that the enzyme(s) responsible for the cleavage in the N-terminal part are metalloprotease(s).[9] In conclusion, degradation studies in spinal cord membranes in the presence of different protease inhibitors indicate that an *o*-phenanthrolin and

phosphoramidon sensitive zinc-metalloprotease(s) is mainly responsible for the degradation of GAL(1–16) and GAL(1–29), since both *o*-phenanthrolin in a concentration dependent manner and phosphoramidon are able to substantially prolong their half-lives.

Acknowledgement

We would like to thank Carina Palmberg who performed the PITC amino acid analysis (at the lab. of Prof. Hans Jörnvall, Department of Physiological Chemistry, Karolinska Institutet). This study was supported by the Swedish MRC (07913, 04X-2887), the Bank of Sweden Tercentenary Foundation, Marcus och Amalia Wallenbergs Minnesfond, Astra Pain Control AB, Lars Hiertas Minnes Stiftelse, Trion FUAB, Konung Gustav V:s och Drottning Victorias Stiftelse and Marianne och Marcus Wallenbergs Stiftelse, Wenner-Gren Foundation and Ivar Bendixsons Foundation.

References

1. Tatemoto, K., Rökaeus, A., Jörnvall, H., McDonald, T. J. and Mutt, V. Galanin — a novel biologically active peptide from porcine intestine. FEBS Lett. 1983; 164: 124–128.
2. Ch'ng, J. L., Christofides, N. D., Anand, P. et al. Distribution of galanin immunoreactivity in the central nervous system and the responses of galanin-containing neuronal pathways to injury. Neuroscience 1985; 16: 343–354.
3. Skofitsch, G. and Jacobowitz, D. M. Galanin-like immunoreactivity in capsaicin sensitive sensory neurons and ganglia. Brain Res. Bull. 1985; 15: 191–195.
4. Wiesenfeld-Hallin, Z., Villar, M. J. and Hökfelt, T. The effects of intrathecal galanin and C-fiber stimulation on the flexor reflex in the rat. Brain Res. 1989; 486: 205–213.
5. Xu, X. J., Wiesenfeld-Hallin, Z. and Hökfelt, T. Intrathecal galanin blocks the prolonged increase in spinal cord flexor reflex excitability induced by conditioning stimulation of unmyelinated muscle afferents in the rat. Brain Res. 1991; 541: 350–353.
6. Wiesenfeld-Hallin, Z., Xu, X. J., Villar, M. J. and Hökfelt, T. Intrathecal galanin potentiates the spinal analgesic effect of morphine: electrophysiological and behavioural studies. Neurosci. Lett. 1990; 109: 217–221.
7. Wiesenfeld-Hallin, Z., Xu, X. J., Hughes, J., Horwell, D. C. and Hökfelt, T. PD134308, a selective antagonist of cholecystokinin type B receptor, enhances the analgesic effect of morphine and synergistically interacts with intrathecal galanin to depress spinal nociceptive reflexes. Proc. Natl. Acad. Sci. U.S.A. 1990; 87: 7105–7109.
8. Rossowski, W. J., Rossowski, T. M., Zacharia, S., Ertan, A. and Coy, D. H. Galanin binding sites in rat gastric and jejunal smooth muscle membrane preparations. Peptides 1990; 11: 333–338.
9. Land, T., Langel, Ü. and Bartfai, T. Hypothalamic degradation of galanin(1–29) and galanin(1–16): identification and characterization of the peptidolytic products. Brain Res. 1991; 558: 245–250.
10. Langel, Ü., Land, T. and Bartfai, T. Design of chimeric peptide ligands to galanin receptors and substance P receptors. Int. J. Pept. Protein. Res. 1992; 39: 516–522.
11. Xu, X. J., Wiesenfeld-Hallin, Z., Fisone, G., Bartfai, T. and Hökfelt, T. The N-terminal 1–16, but not C-terminal 17–29, galanin fragment affects the flexor reflex in rats. Eur. J. Pharmacol. 1990; 182: 137–141.
12. Rigler, R., Wennerberg, A., Cooke, R. M. et al. On the solution structure of galanin. In: Hökfelt, T. (ed). Galanin: a multifunctional peptide in the neuroendocrine system. London: McMillan Press, 1991: 17–25.
13. Kulinski, T., Wennerberg, A. and Rigler, R. Side chain motions and end to end distance distribution in a-helical peptides. In: Laser Spectroscopy of Biomolecules 1992; 1921: 203–208.

ELSEVIER

Molecular Brain Research 29 (1995) 147–156

MOLECULAR BRAIN RESEARCH

Research report

Regulation of mRNA expression involved in Ras and PKA signal pathways during rat hypoglossal nerve regeneration

Sumiko Kiryu, Naonori Morita, Kohji Ohno, Hiroshi Maeno, Hiroshi Kiyama *

Department of Neuroanatomy, Biomedical Research Center, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan

Accepted 11 October 1994

Abstract

Using in situ hybridization histochemistry and immunohistochemistry, the present study examines the cooperative regulation of transcription of molecules involved in the Ras-signal and the cAMP dependent protein kinase (PKA) pathways during peripheral nerve regeneration in rats. Injury to hypoglossal motor neurons resulted in an increase in extracellular regulated kinase (ERK, or MAP kinase) and ERK kinase (MEK, or MAP kinase kinase) mRNAs, but in a decrease in the expression of the catalytic subunits of PKA (Cα and Cβ) mRNAs. These results show the importance of the Ras-signal pathway in the nerve regeneration process and extend recent observation which suggested a cross-talk between the Ras and PKA pathways in vitro. The down-regulation of PKA may facilitate the activation of the Ras pathway which is located downstream of the growth factor receptor. The present study may suggest a possibility of regulatory talk between these two major signal transduction pathways.

Keywords: MAP kinase; ERK; MEK; PKA; MAPKK; Axotomy; Injury

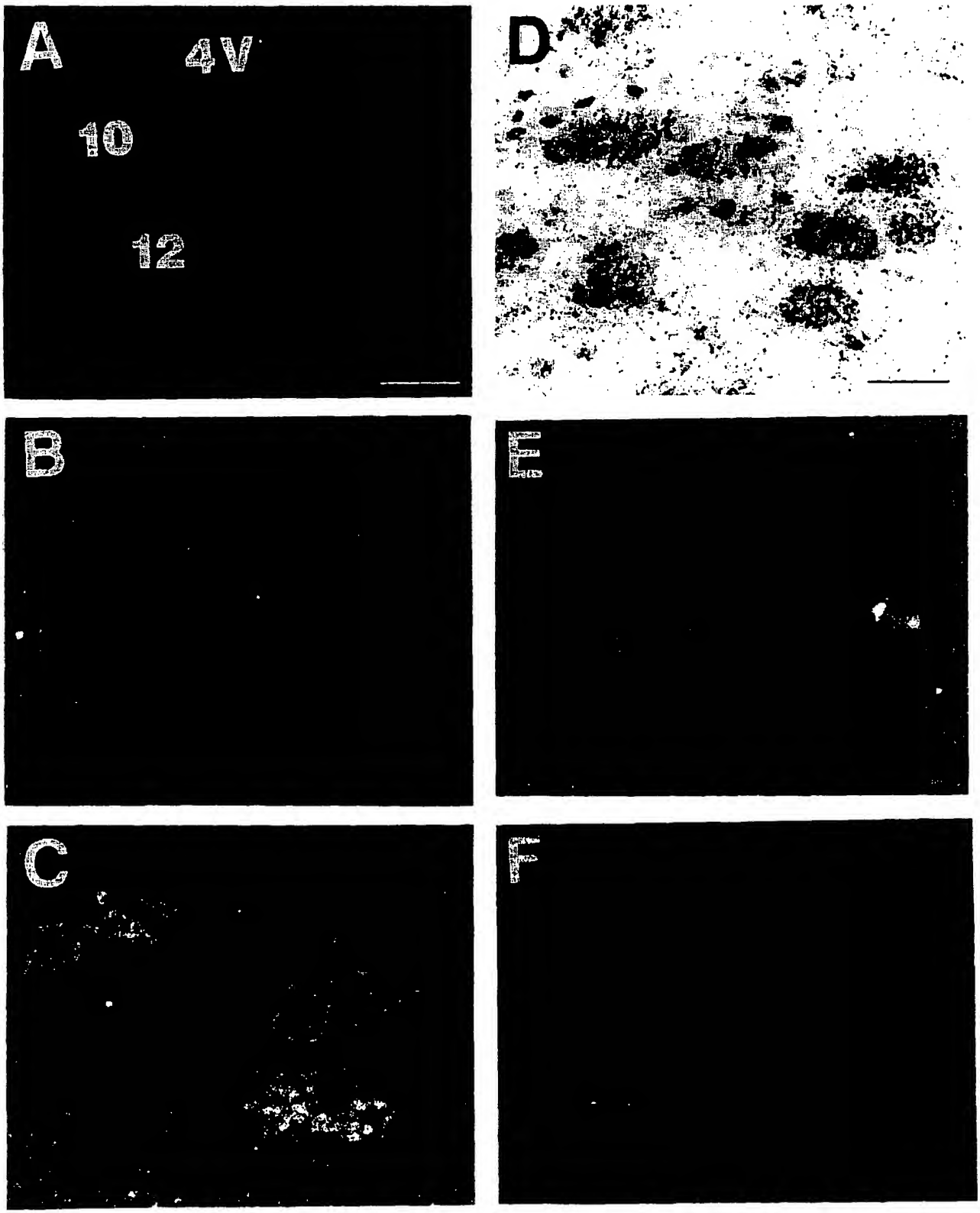
1. Introduction

Recently the possibility of cross-talk between the two major signal transduction pathways namely, the Ras and cAMP dependent protein kinase (PKA) pathways, has been demonstrated in some mammalian cell lines [7,10,16,38,45]. The Ras pathway is a downstream effector pathway following growth factor receptor activation [13]. A receptor tyrosine kinase, which is common among the growth factor receptors, conveys information from the extracellular signal mediator to the extracellular signal regulated kinase (ERK or MAP kinase) through Grb2-SOS, Ras-GTP, Raf-1 and MAP kinase kinase (MAPKK or MEK) cascade to transcription factors [31,39,44]. Among implicated cross-talk points, inhibition of Raf-1 by PKA could be a major element. In addition, phosphorylation of Rap and SOS by PKA has also been suggested to inhibit Ras signal transduction [7,10,16,38,45].

Recently we have focused our attention on the expression of genes associated with nerve regeneration in particular the cytoplasmic membrane associated enzymes phospholipase C (PLC), PKC isozymes and phosphatidylinositol kinase isozymes (PI3K and PI4K). We have reported previously the regulation characteristics of PLC isozymes [34]; the down-regulation of PLCβ and PI4K mRNAs following nerve injury suggested that the signal transduction pathway involving a G-linked receptor was perturbed following damage. However, expression of PLCα, whose structure is different from the other PLC isozymes, was up-regulated, whilst expression of PLCγ and PI3K were unaffected. As PLCγ and PI3K are associated the receptor tyrosine kinase, we then expected an importance of remaining major pathway downstream of the receptor tyrosine kinase namely, the Ras-pathway to see whether it is activated or not following peripheral nerve injury. In addition, we also examined the expression of the PKA genes, catalytic subunits of PKA (PKAC) and regulatory subunits of PKA (PKAR), to determine how these are influenced by damage and regeneration, as the PKA could influence the Ras-signal pathway. A PKA molecule consists of two catalytic (C) and two regulatory (R) subunits. When the catalytic subunits are bound to the regulatory subunit, the enzyme is silent, whereas cAMP can dissociate the C and R subunits' complex and activate the enzyme [37]. Fur-

* Corresponding author. Fax: (81) (6) 879-3589.

SSDI 0169-328X(94)00241-X


A
4V
10
12
D
B
E
C
F

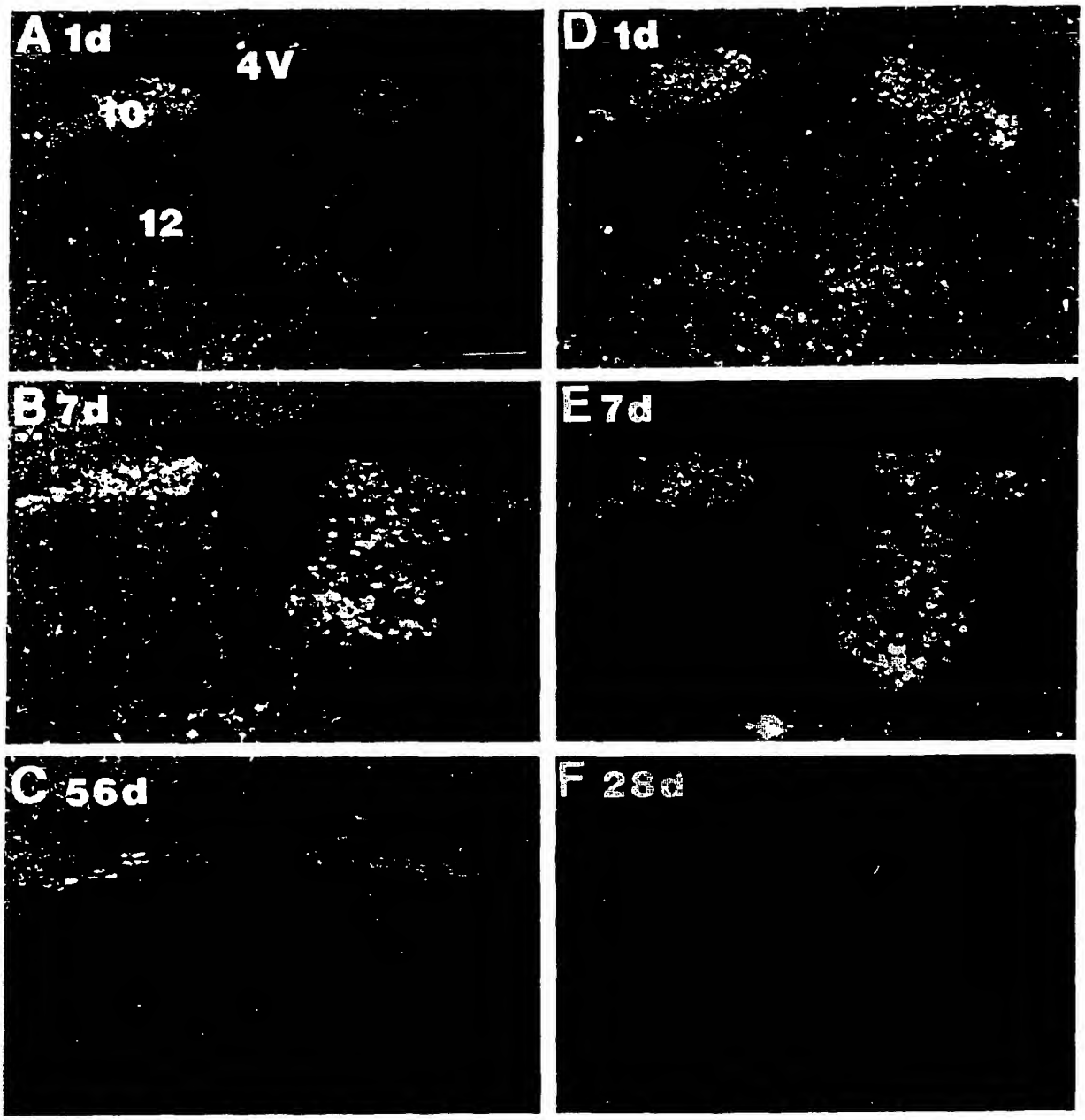


Fig. 2. Expression of ERK1 mRNA in the hypoglossal nucleus 1 day (A), 7 days (B), and 56 days (C) after unilateral hypoglossal nerve resection (right side), and 1 (D), 7 (E), and 28 (F) days after nerve crush (right side). 4V, fourth ventricle; 10, dorsal nucleus of vagus nerve; 12, hypoglossal nucleus. Bar = 200 μm.

thermore, PKA can be classified into two subsets (type I and type II) according to structural difference of regulatory subunits (RI and RII). So far two distinct forms (α and β) of each R subunit have been cloned, and also two isoforms of C subunits (α and β) are known to be expressed in the brain. Thus, at least two

Fig. 1. Histological control experiment demonstrating the specificity of the ERK1 probe and photomicrographs showing mRNA for ERK1, ERK2 and ERK3. A series of three adjacent sections were obtained from unilaterally resected animals 3 days after surgery (right side is the resected side). A: is derived from pre-RNase A treatment; and B: shows the results of the competition test derived from hybridization with an excess amount of unlabeled probe. C: shows the normal reaction. D: shows bright-field photomicrographs of ERK1 mRNA in the ipsilateral hypoglossal nucleus 7 days after axotomy. Sections are counterstained with thionin. E,F: show dark-field photomicrographs for ERK2 (E) and ERK3(F) mRNAs, 3 days after unilateral resection. 4V, fourth ventricle; 10, dorsal nucleus of vagus; 12, hypoglossal nucleus. Bar = 200 μm (A,B,C,E,F); Bar = 30 μm (D).

C subunits (Cα and Cβ) and four R subunits (RIα, RIβ, RIIα, and RIIβ) are thought to exist in the brain [8]. In the present study we examined the regulation of gene transcription in all these six subunits (Cα, Cβ, RIα, RIβ, RIIα, and RIIβ) after hypoglossal nerve axotomy.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing about 100 g were anesthetized with pentobarbital (45 mg/kg), positioned supine and their right hypoglossal nerve carefully exposed. The nerve was then either crushed with a pair of fine forceps for 30 s or resected with a pair of scissors and the distal nerve segment (5 mm) removed.

2.2. In situ hybridization

Animals were decapitated at 1, 3, 5, 7, 14, 21, 35, 49, and 56 days after surgery. Their brains were removed quickly and frozen in powdered dry-ice. Twenty micrometer thick sections were cut on a cryostat, thaw-mounted onto 3-aminopropyltriethoxysilane coated slides, and stored at −80°C until used. Sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min at room temperature, rinsed three times in 1×SSC, acetylated in freshly made 100 mM triethanolamine, pH 7, 0.25% acetic anhydride and then dehydrated in a graded ethanol series. Sections were treated with chloroform for 10 min to remove fat from tissue, and immersed in 100% ethanol twice before hybridization. Hybridization was carried out at 42°C overnight. The composition of the hybridization buffer was as follows: 50% deionized formamide, 4×NaCl-sodium citrate buffer, 1×Denhardt's solution, 0.12 M phosphate buffer (pH 7.2), 2.5% tRNA, 10% dextran sulfate, 50 mM dithiothreitol and labeled probes ($6–9\times10^8$ dpm/ml). Hybridized sections were rinsed briefly in 1×SSC to remove hybridization mixture at room temperature, and washed in 1×SSC at 60°C. Sections were then dehydrated in a graded series of ethanol and air-dried prior to exposure to autoradiography film cassette for 1 week, whereafter sections were dipped in Ilford K-5 photoemulsion (Ilford, UK) diluted 6:4 in water. Sections were then exposed for 7 weeks at 4°C, developed in Kodak D19 developer, counterstained with thionin, dehydrated in a graded series of ethanol to xylene, and covershipped before microscopic observation.

2.3. Oligonucleotides

The oligonucleotide probes for ERK1, ERK2 and ERK3 were synthesized complementary to bases (ERK1: 862–903, ERK2: 557–597, ERK3: 1086–1127) of the rat ERK1,2,3 cDNAs [4–6] and (Cα, 151–190; Cβ, 158–196; RIα, 280–327; RIβ, 32–79; RIIα, 1131–1178; RIIβ, 1341–1380) for rat PKA subunits [20,24,27,28,35,36,43]. These probes were labelled with (α-^{35}S)dATP using terminal deoxynucleotidyl transferase, giving a specific activity of 23–28 MBq/mg. Specificity of the ERK1 hybridization signals was confirmed as follows: (1) no appreciable hybridization signal was detected in a competition experiment involving prehybridization of sections with 100-fold unlabelled probe; (2) prior to in situ hybridization, sections were incubated for 1 h at 37°C with a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and pancreatic RNase A (10 mg/ml, Sigma). No hybridization signal was detected under such conditions (Fig. 1). Specificity against PKA RIα, RIβ and RIIα subunit hybridization signals have already been examined in other reports [27]. The sequences of specific probes to RIIβ, Cα, Cβ mRNAs were carefully chosen to rule out the possibility of cross-reaction against mRNAs presently identified for other proteins using computer analysis.

2.4. Relative quantification of ERK1 mRNA

The number of grains resulting from in situ hybridization was counted by image analysis. The relative area occupied by autoradiographic grains in the hypoglossal nuclei was measured bilaterally on the X-ray film using a computerized image analysis system (MCID; Image Res. Inc., Ontario, Canada). In the same section, we calculated the difference in the optical density between the right (ipsilateral side) and the left (contralateral side) hypoglossal nuclei. For statistical analysis, at least four sections from three rats per time point were studied during same post operative interval. The t-test was done when necessary.

2.5. Immunohistochemistry

Animals were perfused 1, 2, 3, 5, 7, 14 days postoperatively with saline followed by 4% paraformaldehyde containing 14% of a saturated picric acid solution in phosphate buffer. Brains were postfixed in the same fixative for 1–2 days, and then immersed in phosphate buffered saline (PBS) containing 20% sucrose, before being sectioned (20 μm) on a cryostat. Sections were processed with a Vectastain ABC kit (Vector, USA). The primary antisera against ERK1 and MEK1 were obtained from Santa Cruz (California, USA) and UBI (NY, USA), and used at a dilution of 1:1000 and 1:2000, respectively. After overnight incubation in the diluted antisera, sections were then incubated in biotinylated anti rabbit immunoglobulin G followed by an avidin-biotin-horseradish peroxidase complex and rinsed after both incubations in 0.02 M phosphate buffer and finally reacted with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide to reveal a brown reaction product.

3. Results

3.1. ERKs

ERK2 and ERK3 mRNAs were not changed following hypoglossal nerve injury, while only ERK1 mRNA

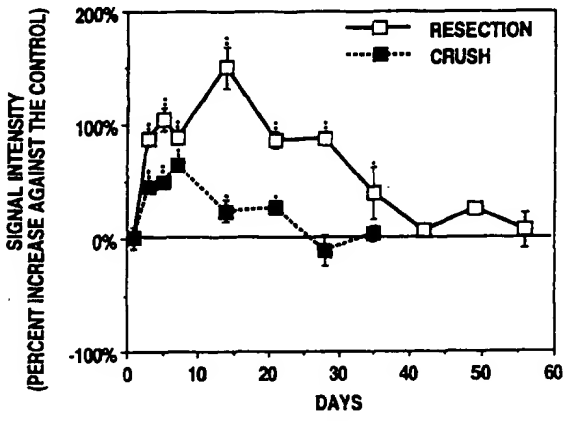


Fig. 3. Changes in ERK1 mRNA levels after hypoglossal nerve resection (open square) and nerve crush (black square). Each point shows the average and standard deviation of the relative signal increases seen in the ipsilateral nucleus when compared to the contralateral side.

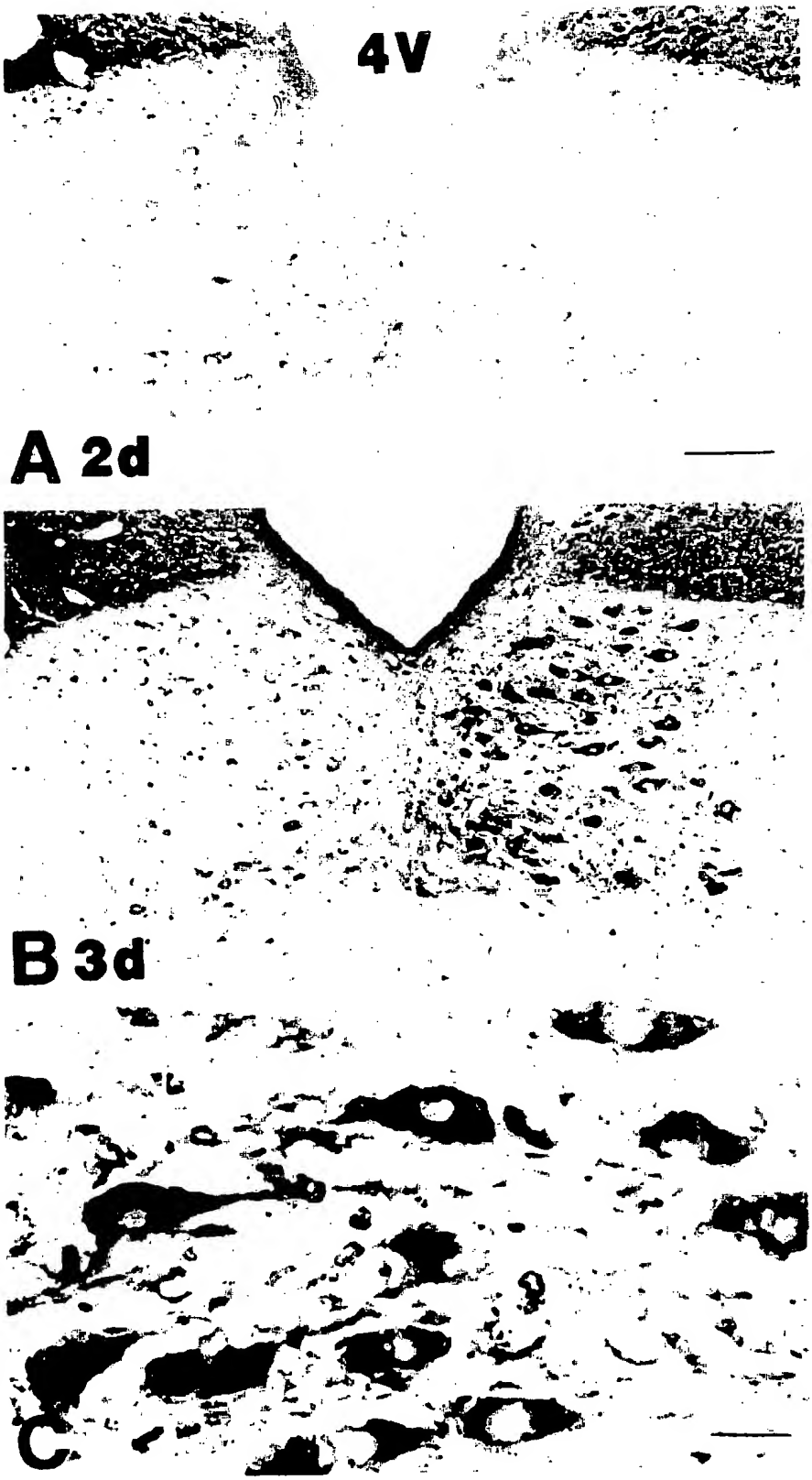


Fig. 4. Bright-field photomicrographs showing ERK1-like immunoreactivity in the hypoglossal nucleus unilaterally operated (right is the operated side) 2 days (A) and 3 days (B) after hypoglossal nerve resection. C: shows higher magnification of the ipsilateral hypoglossal nucleus 3 days after operation. 4V, fourth ventricle. Bar = 120 μm (A,B); Bar = 27 μm (C).

Table 1
Expression profile of MEK1, ERK1, PKA-Cα and PKA-Cβ after the motor nerve resection

	1 day	2 days	3 days	5 days	7 days	14 days	28 days	35 days
MEK1	+/−	+	++	++	++	++	+	+
ERK1	−	−	+	+++	+++	+++	+++	++
ERK1(mRNA)	−	+	++	+++	+++	+++	+++	++
PKACα(mRNA)	+++	nd	+	+	+	+	++	+++
PKACα(mRNA)	+++	nd	+	+	+	+	++	+++

The number of + indicates the extent of MEK1, ERK1, PKA Cα and Cβ mRNA expression on ipsilateral hypoglossal nucleus, compared with background level. − shows that the mRNA level is almost the background level. nd, not determined.

was found to be up-regulated in the hypoglossal motor neurons but not in glia (Figs. 1 and 2). ERK1 mRNA transiently increased after both nerve resection and crush. The increase in ERK1 hybridization signal was detected initially in the ipsilateral hypoglossal nucleus 2 days after either surgical procedure, however, the intensity of the hybridization signal markedly increased to a peak level during the following 3 days and persisted at this level for 14 days for the resection rats and 7 days for the nerve crushed rats. Thereafter, the hybridization signal gradually decreased until control levels were reached during the following 6 weeks for the resection group and 3 weeks for the nerve crush group (Figs. 2 and 3). The increase in ERK1 mRNA was greater following nerve resection (Fig. 3). A similar expression profile was seen for ERK1 protein; the increase in ERK1 mRNA was paralleled by an increase in ERK1 like immunoreactivity (Fig. 4). The increase in ERK1-like immunoreactivity was first detected in injured motoneurons 3 days after surgery; that is one day after the initial increase in ERK1 mRNA (Table 1). While ERK has been detected previously in both the cytoplasm and nucleus in some cell lines, in this study ERK1 was observed mainly in the cytoplasm after nerve injury.

3.2. MEK

MEK is a kinase which is located upstream of ERK in the Ras signal transduction cascade. Expression of this molecule after nerve injury was examined by immunohistochemistry. An increase in the intensity of MEK-like immunoreactivity was detected in the ipsilateral motoneurons 2 days after nerve transection (Fig. 5). Appearance of MEK immunoreactivity was detected one day earlier than the downstream protein, ERK (Table 1). The quicker increase of MEK immunoreactivity than ERK-1 immunoreactivity is probably reasonable for the facilitation of the signal cascade, and also implies that MEK up-regulation was not due to the up-regulation of ERK which can phosphorylate MEK.

3.3. PKA

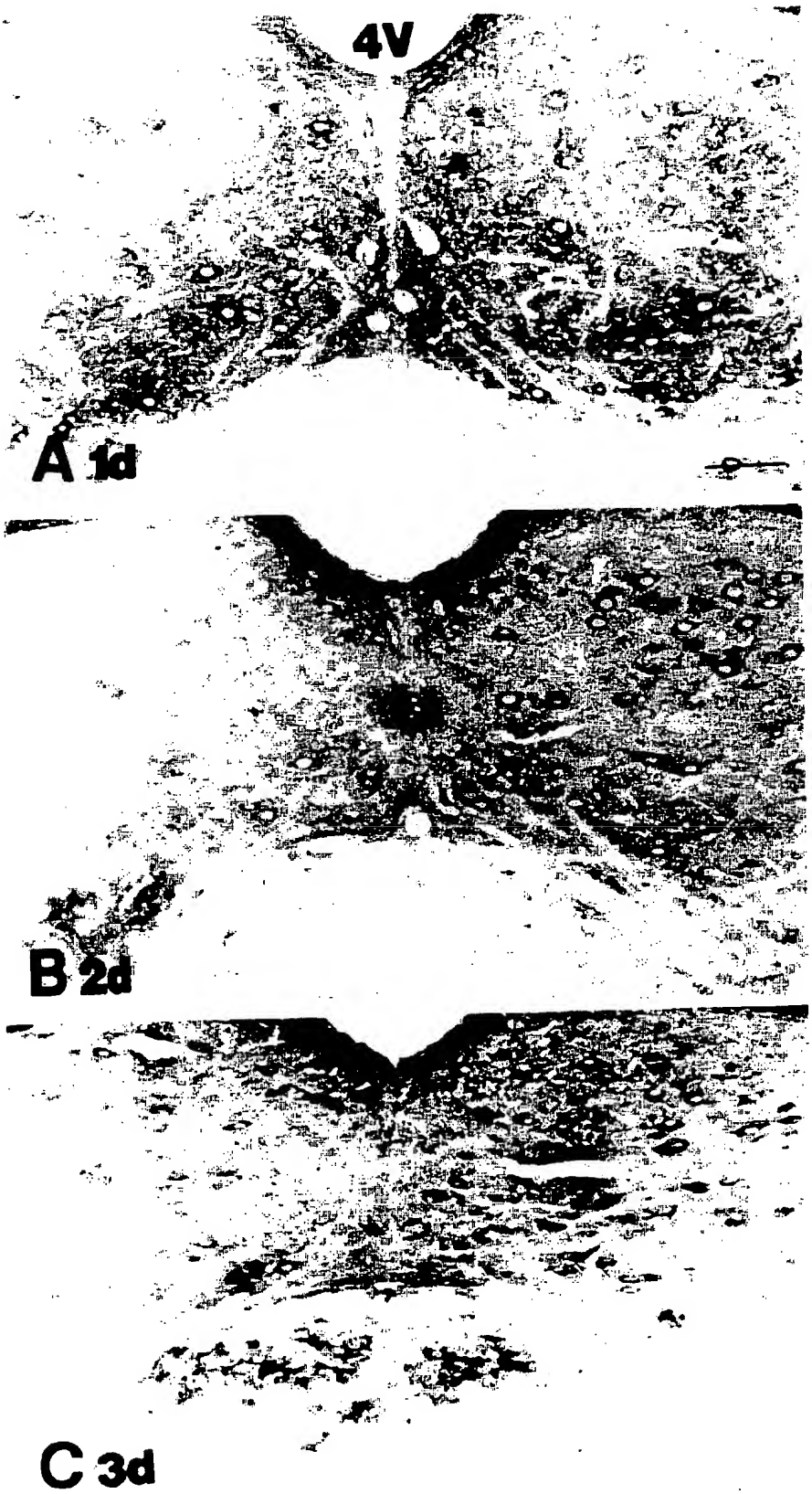
In the hypoglossal nucleus of normal rats, the PKA Cα and Cβ subunit mRNAs are expressed in abundance, while a moderate level of RIα, β and RIIα, β subunit mRNAs are detected. After resection of the hypoglossal nerve, no dramatic change was seen for either of PKA regulatory subunits; rather a slight increase in the RIIα and RIIβ subunit mRNAs was observed in the ipsilateral hypoglossal motor neurons, and expression of RIα and RIβ remained unchanged following nerve injury. In contrast, a marked decrease in Cα and Cβ mRNAs was observed (Fig. 6). The decrease in these mRNAs was detected initially 3 days after surgery, with a plateau being reached about 5–7 days after the operation. Recovery of Cα and Cβ mRNA levels to control levels was seen 4–5 weeks after the operation. The duration of Cα and Cβ down-regulation was shorter than that observed for ERK and MEK. The time of on-set of this decreased gene expression was similar to that of ERK1.

4. Discussion

The present study demonstrates that during hypoglossal nerve regeneration, ERK1 mRNA and immunoreactivity, which is a pivotal enzyme in the Ras-signal pathway, are up-regulated. Further, an earlier up-regulation of MEK, which is a ERK regulating enzyme, was also found. It is feasible to expect that the up-regulation of this pathway is important for peripheral nerve regeneration which can be effected by some growth factors. In addition, the down-regulation of the PKA system may facilitate the up-regulation of MEK and ERK activity.

Several lines of studies have reported the up-regulation of ERK activity after stimulation of various kinds of growth factors [1,2,4–6,9,15,21,26,30,32,33,40,42]. Two kinds of activation profiles have been described among growth factors: the EGF type which shows an early transient up-regulation, and the

Fig. 5. Bright-field photomicrographs showing MEK-like immunoreactivity in the hypoglossal nucleus 1 day (A), 2 days (B), and 3 days (C) after hypoglossal nerve resection (right hand side is the operated side). 4V, fourth ventricle. Bar = 120 μm.


4V
A 1d
B 2d
C 3d

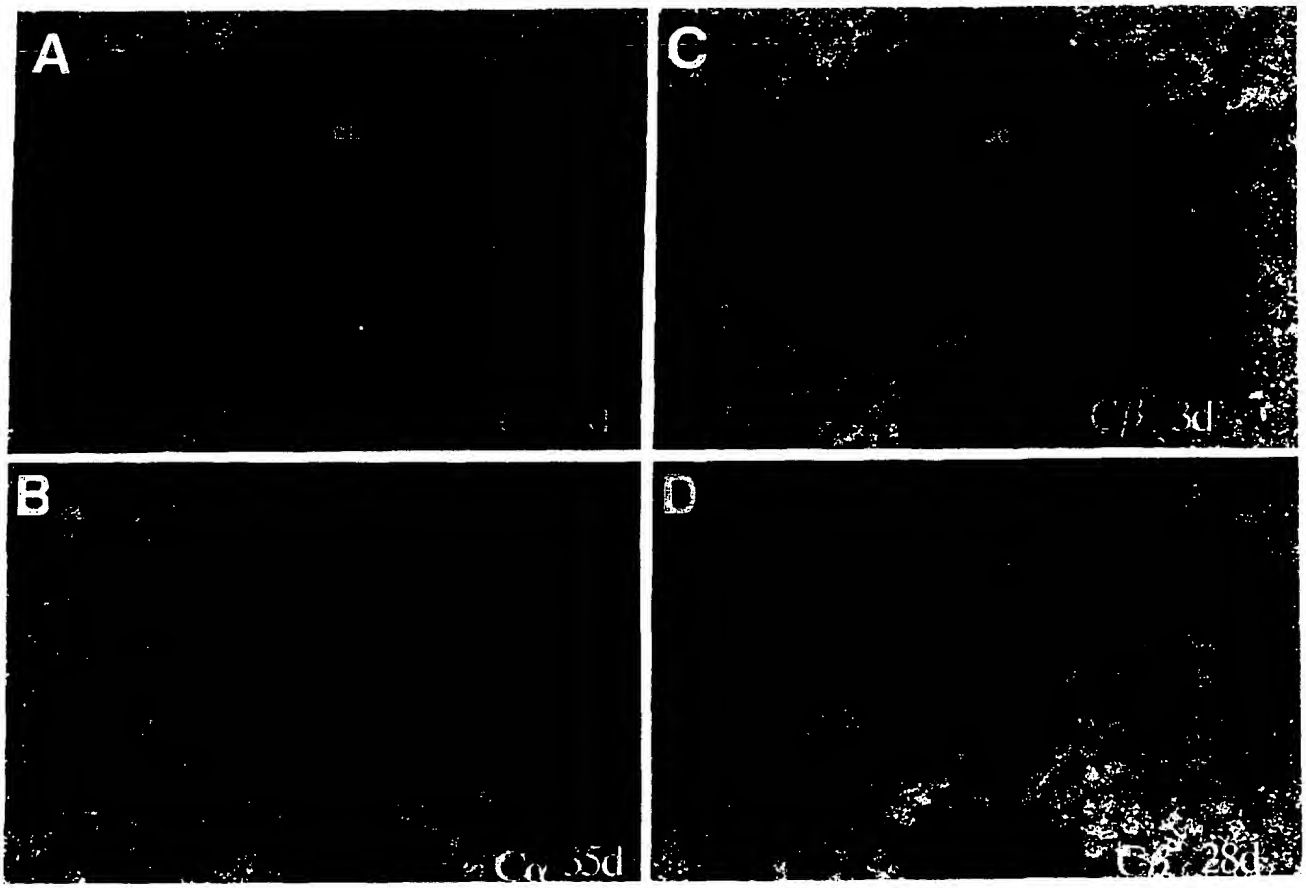


Fig. 6. Dark-field photomicrographs showing mRNA for Cα and Cβ subunit of PKA in hypoglossal nucleus 3 days (A,C), 28 days (D) and 35 days (B) after axotomy. Both Cα and Cβ mRNA levels in the ipsilateral side (right) decrease 3 days after operation, and recover to the normal level around 28 days (crush, D) and 35 days (resection B) after operation. cc; central canal.

NGF/FGF type which shows biphasic activation comprising of an early and a late component [9,15,21,29, 30,33]. The late component is supposed to be due to newly synthesized protein. Therefore, the NGF/FGF type of growth factors may promote ERK transcription. Indeed, cranial and spinal motor neurons express numerous growth factor receptors including FGFR1 (flg, FGF receptor), TrkB (high affinity BDNF receptor), $p75^{NGFR}$ (low affinity component of NGF/BDNF/ NT-3) [12,14,19,22,41]. Among these receptors, Trk B and $p75^{NGFR}$ have been demonstrated to be up-regulated after nerve injury. As for TrkB, this receptor is known to have two distinct forms, one is so-called the truncated type which does not have an intracellular tyrosine kinase domain, and the other is the non-truncated type which contains full length of the molecules including the tyrosine kinase domain. Interestingly, transcription of the truncated type of the receptor was down-regulated after motor nerve injury, whereas expression of the non-truncated type of the receptor was up-regulated [14]. This opposing regulation of TrkB can lead to an increase in the strength of the signal transduction pathway initiated by activation of the TrkB receptor by BDNF. As for the growth factors themselves, up-regulation of the synthesis of NGF and BDNF has been shown in lesioned motoneurons and/or Schwann cells [11,17,18,22]. Furthermore, exogenous application of BDNF to motor nerve lesion increased the survival of the motor neurons [19,23]. Taking these evidence into account, it is likely that BDNF is probably released from both the injured motor neurons and surrounding Schwann cells, binds to its receptor (non-truncated TrkB) in an autocrine manner and up-regulates the transcription of MEK and ERK to accelerate the following Ras signaling pathway. This series of events involving growth factors, their receptors and intracellular signaling molecules along the Ras signal transduction pathway, might be crucial in facilitating neuronal regeneration.

In addition to growth factors, some factors from other signal pathways have been suggested to activate ERK, for example G protein coupled PLC. Recent studies by Meloche et al. and L'Allemain et al. [25,29,30] reported that pertussis toxin inhibited the α-thrombin induced activation of both ERK1 ($p44^{mapk}$) and ERK2 ($p42^{mapk}$). α-Thrombin is known to activate

PLC by interacting with pertussis toxin-sensitive and -insensitive G proteins and to inhibit adenylyl cyclase through pertussis toxin-sensitive Gi proteins [42]. This suggests that the activation of the adenylyl cyclase may lead to an inhibition of ERK1 probably via activation of a PKA, as recent reports have suggested that the implied and the PKA pathways may cross-talk. PKA has been shown to inhibit Raf-1 activity which is located up-stream of the MEK. In addition, PKA was also shown to phosphorylate SOS and Rap. The phosphorylation of SOS inhibits the signal pathway between Grb2 and Ras-GTP, while Rap phosphorylation causes a competition between Ras-GTP and Rap-GTP leading to inhibition of Raf-1 activation which is adjacent to Ras-GTP in the Ras pathway [3]. At all junctures in the pathway, PKA seems to have an inhibitory influence on the various components of the Ras-signal pathway. The results detailed above were derived from in vitro studies using selective mammalian cell lines, and so the functional significance of these interactions in vivo has been unknown. In the present study, we have suggested that the down-regulation of the PKA catalytic subunits ($C\alpha$ and $C\beta$) possibly disinhibited the PKA inhibition of Ras-signal pathway leading to a facilitation of the Ras-signal pathway. Therefore, at least some of the molecules involved in these two major signal pathways are thought to be regulated cooperatively during the peripheral nerve regeneration process. These findings may suggest that linkage between the Ras-signal pathway and the PKA pathway is of importance for the facilitation of the Ras-signaling pathway during nerve regeneration.

Acknowledgements

We are grateful to Dr. Masaya Tohyama (Osaka University Medical School, Japan.) for support and encouragement, and Drs. P.C. Emson and S.J. Augood (MRC, Babraham, Cambridge, UK) for critical reading and English correction. This work was supported by Senri Life Science Foundation (Osaka, Japan), Brain Science Foundation (Tokyo, Japan) and Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

[1] Ahn, N.G. and Krebs, E.G., Evidence for an epidermal growth factor-stimulated protein kinase cascade in swiss 3T3 cells, *J. Biol. Chem.*, 265 (1990) 11495–11501.

[2] Ahn, N.G., Weiel, J.E., Chan, C.P. and Krebs, E.G., Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from swiss 3T3 cells, *J. Biol. Chem.*, 265 (1990) 11487–11494.

[3] Bokoch, G.M., Biology of the Rap proteins, members of the *ras* superfamily of GTP-binding proteins, *Biochem. J.*, 289 (1993) 17–24.

[4] Boulton, T.G. and Cobb, M.H., Identification of multiple extracellular signal-regulated kinases (ERKs) with antipeptide antibodies, *Cell Regul.*, 2 (1991) 357–371.

[5] Boulton, T.G., Nye, S.H., Robbins, D.J., Ip, N.Y., Radziejewska, E., Morgenbesser, S.D., DePinho, R.A., Panayotatos, N., Cobb, M.H. and Yancopoulos, G.D., ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF, *Cell*, 65 (1991) 663–675.

[6] Boulton, T.G., Yancopoulous, G.D., Gregory, J.S., Slaughter, C., Moomaw, C., Hsu, J. and Cobb, M.H., An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control, *Science*, 249 (1990) 64–67.

[7] Burgering, B.M., Pronk, G.J., Weeren, P.C.v., Chardin, P. and Bos, J.L., cAMP antagonizes p21ras-directed activation of extracellular signal-regulated kinases 2 and phosphorylation of mSos nucleotide exchange factor, *EMBO J.*, 12 (1993) 4211–4220.

[8] Cadd, G. and McNight, S., Distinct patterns of cAMP-dependent protein kinase gene expression in mouse brain, *Neuron*, 3 (1989) 71–79.

[9] Chao, M.V., Growth factor signaling: where is the specificity?, *Cell*, 68 (1992) 995–997.

[10] Cook, S.J. and McCormick, F., Inhibition by cAMP of Ras-dependent activation of Raf, *Science*, 262 (1993) 1069–1072.

[11] Eckenstein, F.P., Shipley, G.D. and Nishi, R., Acidic and basic fibroblast growth factors in the nervous system: distribution and differential alternation of levels after injury of central versus peripheral nerve, *J. Neurosci.*, 11 (1991) 412–419.

[12] Ernfors, P., Rosario, C.M., Merlio, J.-P., Grant, G., Aldskogius, H. and Persson, H., Expression of mRNAs for neurotrophin receptors in the dorsal root ganglion and spinal cord during development and following peripheral or central axotomy, *Mol. Brain Res.*, 17 (1993) 217–226.

[13] Feig, L.A., The many roads that lead to Ras, *Science*, 260 (1993) 767–768.

[14] Frisen, J., Verge, V.M.K., Fried, K., Risling, M., Persson, H., Trotter, J., Hökfelt, T. and Lindholm, D., Characterization of glial trkB receptors: differential responses to injury in the central and peripheral nervous systems, *Proc. Natl. Acad. Sci. USA*, 90 (1993) 4971–4975.

[15] Gotoh, Y., Nishida, E., Yamashita, T., Hoshi, M., Kawakami, M. and Sakai, H., Microtubule-associated-protein(MAP) kinase activated by nerve growth factor and epidermal growth factor in PC12 cells, *Eur. J. Biochem.*, 193 (1990) 661–669.

[16] Graves, L.M., Bornfeldt, K.E., Raines, E.W. and Potts, B.C., Protein kinase A antagonizes platelet-derived growth factor-induced signaling by mitogen-activated protein kinase in human arterial smooth muscle cells, *Proc. Natl. Acad. Sci. USA*, 90 (1993) 10300–10304.

[17] Heumann, R., Korsching, S., Bandtlow, C. and Thoenen, H., Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection, *J. Cell Biol.*, 104 (1987) 1623–1631.

[18] Heumann, R., Lindholm, D., Meyer, M., Radeke, M., MIsko, T., Shooter, E. and Thoenen, H., Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: role of macrophages, *Proc. Natl. Acad. Sci. USA*, 84 (1987) 8735–8739.

[19] Hughes, R.A., Sendtner, M. and Thoenen, H., Members of several gene families influence survival of rat motoneurons in vitro and in vivo, *J. Neurosci. Res.*, 36 (1993) 663–671.

[20] Jahnsen, T., Hedin, L., Kidd, V.J., Beattie, W.G., Lohmann, S.M., Walter, U., Durica, J., Schiltz, T.Z., Browner, M., Lowrence, C.B., Goldman, D., Ratoosh, S.L. and Richards, J.S.,

Molecular cloning, cDNA structure and regulation of the regulatory subunit (RII51) of type I cAMP-dependent protein kinase from rat granulosa cells, *J. Biol. Chem.*, 261 (1986) 12352–12361.

[21] Kahan, C., Seuwen, K., Meloche, S. and Pouyssegur, J., Coordinate, biphasic activation of p44 mitogen-activated protein kinase and S6 kinase by growth factors in hamster fibroblasts, *J. Biol. Chem.*, 267 (1992) 13369–13375.

[22] Kobayashi, N.R., Bedard, A.M. and Tetzlaff, W., Increased expression of trkB and BDNF mRNA in rat facial motoneurons after axotomy, *Soc. Neurosci. Abstr.*, 19 (1993) 110.10.

[23] Koliatsos, V., Clatterbuck, R.E., Winslow, J.W., Cayouette, M.H. and Price, D.L., Evidence that brain-derived neurotrophic factor is a trophic factor for motor neurons in vivo, *Neuron*, 10 (1993) 359–367.

[24] Kuno, T., Ono, Y., Hirai, M., Hashimoto, S., Shuntoh, H. and Tanaka, C., Molecular cloning and cDNA structure of the regulatory subunit of type I cAMP-dependent protein kinase from rat brain, *BBRC*, 146 (1987) 878–883.

[25] L'Allemain, G., Pouysségur, J. and Weber, M.J., p42/mitogen-activated protein kinase as a converging target for different growth factor signaling pathways: use of pertussis toxin as a discrimination factor, *Cell Regul.*, 2 (1991) 675–684.

[26] Loeb, D.M., Tsao, H., Cobb, M.H. and Greene, L.A., NGF and other growth factors induce an association between ERK1 and the NGF receptor, gp140^{prtrk}, *Neuron*, 9 (1992) 1053–1065.

[27] Lonnerberg, P., Parvinen, M., Jahnsen, T., Hansson, V. and Persson, H., Stage- and cell-specific expression of cyclic adenosine 3',5'-monophosphate-dependent protein kinases in rat seminiferous epithelium, *Biol. Reprod.*, 46 (1992) 1057–1068.

[28] Massa, J., Fellow, R. and Maurer, R., Rat RIβ isoform of type I regulatory subunit of cyclic adenosine monophosphate-dependent protein kinase: cDNA sequence analysis, mRNA tissue specificity, and rat/mouse difference in expression in testis, *Mol. Reprod. Dev.*, 26 (1990) 129–133.

[29] Meloche, S., Pagés, G. and Pouysségur, J., Functional expression and growth factor activation of an epitope-tagged p44 mitogen-activated protein kinase, p44^{mapk}, *Mol. Biol. Cell*, 3 (1992) 63–71.

[30] Meloche, S., Seuwen, K., Pagés, G. and Pouysségur, J., Biphasic and synergistic activation of p44^{mapk} (ERK1) by growth factors: activation and mitogenicity, *Mol. Endo.*, 6 (1992) 845–854.

[31] Moodie, S.A., Willumsen, B.M., Weber, M.J. and Wolfman, A., Complexes of Ras·GTP with Raf-1 and mitogen-activated protein kinase kinase, *Science*, 260 (1993) 1658–1661.

[32] Peraldi, P., Scimeca, J.C., Filloux, C. and Van, O.E., Regulation of extracellular signal-regulated protein kinase-1 (ERK-1; pp44/mitogen-activated protein kinase) by epidermal growth factor and nerve growth factor in PC12 cells: implication of ERK1 inhibitory activities, *Endocrinology*, 132 (1993) 2578–85.

[33] Qiu, M.-S. and Green, S.H., PC12 cell neuronal differentiation is associated and consequent prolonged ERK activity, *Neuron*, 9 (1992) 705–717.

[34] Saika, T., Kiyama, H., Matsunaga, T. and Tohyama, M., Differential regulation of phospholipase C isozymes in the rat facial nucleus following axotomy, *Neuroscience*, 59 (1994) 121–129.

[35] Sandberg, M., Levy, F.O., Oyen, O., Hansson, V. and Jahnsen, T., Molecular cloning, cDNA structure and deduced amino acid sequence for the hormone-induced regulatory subunit (RII β) of cAMP-dependent protein kinase from rat ovarian granulosa cells, *BBRC*, 154 (1988) 705–711.

[36] Scott, D.J., Glaccum, M.B., Zoller, M.J., Uhler, M.D., Helfman, O.M., McNight, G.S. and Krebs, E.G., The molecular cloning of a type II regulatory subunit of the cAMP dependent protein kinase from rat skeltal muscle and mouse brain, *Proc. Natl. Acad. Sci. USA*, 84 (1987) 5192–5196.

[37] Scott, J.D., Cyclic nucleotide-dependent protein kinases, *Pharmacol. Ther.*, 50 (1991) 123–145.

[38] Sevetson, B.R., Kong, X., John, J. and Lawrence, C., Increasing cAMP attenuates activation of mitogen-activated protein kinase, *Proc. Natl. Acad. Sci. USA*, 90 (1993) 10305–10309.

[39] Thomas, S.M., DeMarco, M., D'Arcangelo, G., Halegoua, S. and Brugge, J.S., Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases, *Cell*, 68 (1992) 1031–1040.

[40] Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P., Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor, *Biochem. J.*, 288 (1992) 351–355.

[41] Verge, V.M.K., Merlio, J., Grondin, J., Ernfors, P., Persson, H., Riopelle, R.J., Hökfelt, T. and Richardson, P.M., Colocalization of NGF binding sites, trk mRNA, and low-affinity NGF receptor mRNA in primary sensory neurons: responses to injury and infusion of NGF, *J. Neurosci.*, 12 (1992) 4011–4022.

[42] Vouret, C.V., Van, O.S.E., Scimeca, J.C., Van, O.E. and Pouyssegur, J., Differential activation of p44^{mapk} (ERK1) by α-thrombin and thrombin-receptor peptide agonist, *Biochem. J.*, 289 (1993) 209–214.

[43] Wiemann, S., Voß, H., Kinzel, V. and Pyerin, W., Rat Cα catalytic subunit of the cAMP-dependent protein kinase: cDNA sequence and evidence that it is the only isoform expressed in myoblasts, *Biochem. Biophys. Acta*, 1089 (1991) 254–256.

[44] Wood, K.W., Sarnecki, C., Roberts, T.M. and Blenis, J., ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK, *Cell*, 68 (1992) 1041–50.

[45] Wu, J., Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate, *Science*, 262 (1993) 1065–1068.

Journal of Neurochemistry
Lippincott-Raven Publishers, Philadelphia

Time-Resolved Signaling Pathways of Nerve Growth Factor Diverge Downstream of the p140trk Receptor Activation Between Chick Sympathetic and Dorsal Root Ganglion Sensory Neurons

Franz-Josef Klinz and Rolf Heumann

Department of Molecular Neurobiochemistry, Ruhr-Universität Bochum, Bochum, Germany

Abstract: We have recently shown that the small GTP binding protein p21ras is essential for nerve growth factor (NGF)-mediated survival of peripheral embryonic chick dorsal root ganglia (DRG) sensory but not sympathetic neurons. To investigate at which level of the signaling cascade the pathways diverge, we have studied the time-resolved pattern of NGF-stimulated tyrosine phosphorylation of proteins within 4 h after addition of the neurotrophin. In both chick sympathetic neurons [embryonic day (E) 12] and DRG sensory neurons (E9) NGF induces within 1 min the autophosphorylation of the receptor tyrosine kinase p140trk. However, the pattern of substrate protein tyrosine phosphorylation downstream of p140trk is distinctly different in both neuronal subtypes. In sympathetic neurons, we observe within 1 min the tyrosine phosphorylation of a new substrate protein, p105, reaching maximal levels at 3 min. Tyrosine phosphorylation of p105 remains elevated for up to 4 h. Subsequent to p105, NGF induces the tyrosine phosphorylation of p42, a protein belonging to the family of mitogen-activated protein (MAP) kinases. This stimulation is transient, reaching maximal levels at 10 min and returning to very low levels already after 2 h. In DRG sensory neurons, tyrosine phosphorylation of p105 is weak and very short lived, disappearing already after treatment with NGF for 10 min. In contrast, activation of MAP kinase p42 in DRG sensory neurons is more stable than in sympathetic neurons. All NGF-stimulated tyrosine phosphorylation events were inhibited by preincubation of neurons with the tropomyosin-related kinase (trk) inhibitor K252a. We suggest the working hypothesis that persistent tyrosine phosphorylation of p105 may play a role in the p21ras-independent NGF survival pathway of chick sympathetic neurons. **Key Words:** Chick sympathetic neurons—Chick dorsal root ganglia sensory neurons—Nerve growth factor signal transduction—Protein tyrosine phosphorylation—Tropomyosin-related kinase receptor—Mitogen-activated protein kinase.
J. Neurochem. **65,** 1046–1053 (1995).

Nerve growth factor (NGF) belongs to the family of neurotrophins specifically interacting with the receptor tyrosine kinase p140trk and with the low-affinity receptor p75 (see, for review, Meakin and Shooter, 1992; Chao, 1992; Barbacid, 1993). Recent gene knock-out experiments on NGF and on the tyrosine kinase domain of its p140trk receptor have confirmed that NGF activity is essential for the survival of peripheral sympathetic and certain types of sensory neurons involved in pain transmission (see, for review, Snider, 1994). The p75 low-affinity NGF receptor binds to all neurotrophins and appears to modulate the p140trk receptor activity at limiting concentrations of the NGF ligand (Barker and Shooter, 1994). In addition, an intrinsic p75 receptor signaling mechanism involving activation of the sphingomyelin cycle has been described recently (Dobrowsky et al., 1994).

As with other tyrosine kinase receptors, aggregation and tyrosine autophosphorylation of tropomyosin-related kinase (trk) receptor are essential to initiate the various signaling mechanisms of NGF (Jing et al., 1992; Clary et al., 1994). The subsequent steps of intracellular signaling events (see, for review, Heumann, 1994; Saltiel and Decker, 1994) have been extensively studied in transformed rat pheochromocytoma PC12 cells, which differentiate into sympathetic neuron-like cells on long-term exposure to NGF (Greene and Tischler, 1976). The autophosphorylation of the trk receptor opens binding sites for the src homology region 2 (SH2) domains of the SHC protein (Obermeier et al., 1994; Ohmichi et al., 1994; Stephens et al., 1994), the p85 regulatory subunit of phosphati-

Received December 19, 1994; revised manuscript received March 6, 1995; accepted March 14, 1995.

Address correspondence and reprint requests to Dr. F.-J. Klinz at Department of Molecular Neurobiochemistry, Ruhr-Universität Bochum, Universitätsstrasse 150, D-44780 Bochum, Germany.

Abbreviations used: DRG, dorsal root ganglia; E, embryonic day; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; trk, tropomyosin-related kinase.

dylinositol-3 kinase (Ohmichi et al., 1992*a*; Soltoff et al., 1992) and of phospholipase C-γ1 (Vetter et al., 1991; Obermeier et al., 1994; Stephens et al., 1994). During this process these proteins become phosphorylated on tyrosine residues themselves, and additional SH2 domain proteins are recruited to the membrane. After Grb2-mediated membrane recruitment (Hashimoto et al., 1993; Basu et al., 1994), the p21ras exchange factor mSOS is thought to activate in turn p21ras protein, an inner membrane-bound protein oscillating between the inactive GDP-bound and the signaling-competent GTP-bound conformation (see, for review, Boguski and McCormick, 1993). Other membrane-recruited proteins belong to the family of the raf serine/threonine kinases or mitogen-activated protein (MAP) kinase kinase (MEK) kinases (Oshima et al., 1991; Ohmichi et al., 1992*b*; Jaiswal et al., 1994; Lange-Carter and Johnson, 1994; Vaillancourt et al., 1994). Subsequently, these proteins induce the phosphorylation of MEK and finally MAP kinase (Boulton et al., 1991; Ahn et al., 1992; Lloyd and Wooten, 1992; Qiu et al., 1992; Thomas et al., 1992; Wood et al., 1992). p21ras activity and MEK1 activity have recently been shown to be essential for the induction of neurite outgrowth in PC12 cells (Hagag et al., 1986; Szeberenyi et al., 1990; Cowley et al., 1994).

Although the elucidation of the mechanisms leading to neurite outgrowth in PC12 cells is progressing well, surprisingly little is known about the sequence of events coupled to neurite outgrowth and survival in postmitotic neurons. As an initial approach we have shown previously that activated p21ras protein promotes neurite outgrowth and survival in embryonic chick dorsal root ganglia (DRG) sensory but not sympathetic neurons (Borasio et al., 1989, 1993). Measuring the time-resolved NGF-stimulated tyrosine phosphorylation of proteins in these peripheral neural crest-derived subtypes of neurons, we describe here the common activation of p140trk and divergent downstream signaling pathways.

MATERIALS AND METHODS

Materials

NGF from adult male mouse submaxillary glands was purified as described (Suda et al., 1978) and kindly provided by H. Rohrer (Frankfurt, Germany). Recombinant human NGF and NT3 were gifts of Genentech (San Francisco, CA, U.S.A.).

Plastic dishes were from Nunc (Wiesbaden, Germany). Ham's F14 medium and horse serum were obtained from GIBCO (Eggenstein, Germany). Nonidet P-40 was purchased from Boehringer (Mannheim, Germany). Sodium deoxycholate and sodium dodecyl sulfate (SDS) were obtained from Serva (Heidelberg, Germany). Anti-phosphotyrosine monoclonal antibody 4G10 and anti-MAP kinase R2 antiserum were purchased from UBI (Lake Placid, NY, U.S.A.). Anti-trk rabbit antiserum was purchased from Oncogene Science (Uniondale, NY, U.S.A.). The enhanced chemiluminescence detection system (ECL) and Hyperfilm-ECL were obtained from Amersham (Braunschweig, Germany). All other reagents were from Sigma (Deisenhofen, Germany).

Cell culture

Chick embryonic sensory DRG neurons [embryonic day (E) 9] and sympathetic neurons (E12) were isolated from the corresponding ganglia at the indicated ages and cultured using previously described methods (Lindsay et al., 1985). After trypsinization and dissociation, the cell suspension was preplated as described to remove fibroblasts and glial cells. The neuron-enriched cell suspension was plated at Nunc Petri dishes that had been coated with poly-L-lysine hydrobromide (70–150 kDa). The cultures were maintained with F14 medium containing 10% heat-inactivated horse serum at 37°C and 2.5% CO_2 in a humidified environment.

Two hours after plating NGF was added at a concentration of 10 ng/ml. For some experiments, neurons were preincubated for 10 min with the trk kinase inhibitor K252a at a concentration of 1 μM.

Cell lysis

Cells were washed two times with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline and lysed in a small volume of lysis buffer [50 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 40 m*M* NaF, 5 m*M* EDTA, 5 m*M* EGTA, 1 m*M* sodium orthovanadate, 1% (vol/vol) Nonidet P-40, 0.1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, 1 m*M* phenylmethylsulfonyl fluoride, and 10 μg/ml of aprotinin] using a rubber policeman. Lysates were centrifuged for 5 min at 16,000 *g* and 4°C. The supernatant was immediately used for experiments or frozen at −80°C.

SDS-polyacrylamide gel electrophoresis (PAGE)

Three volumes of lysate were mixed with 1 volume of fourfold concentrated sample buffer according to the procedure of Laemmli (1970) and heated for 5 min at 95°C. Proteins in samples together with molecular mass marker proteins were separated on SDS-polyacrylamide gels prepared by the method of Laemmli (1970). If not otherwise indicated, the slab gels (1.5 mm thick) consisted of a 3% stacking gel (10 mm long) and an 8% separating gel (50 mm long).

To analyze the phosphorylation state of MAP kinase p42, we used low-cross-linker SDS-polyacrylamide gels (1.5 mm thick), consisting of a 5% stacking gel (15 mm long) and a 15% separating gel (120 mm long).

Immunoblot analysis

Subsequent to SDS-PAGE, proteins were electrotransferred onto nitrocellulose membranes for 2 h at 100 V in buffer containing 25 m*M* Tris, 192 m*M* glycine, 20% (vol/vol) methanol, and 0.01% (wt/vol) SDS. Proteins on blots were stained with Ponceau S (Nakamura et al., 1985) to ascertain that comparable amounts of protein were loaded in each lane. The nitrocellulose sheets were blocked with 1% (wt/vol) nonfat dry milk, reacted with the primary antibody followed by reaction with the second antibody coupled to horseradish peroxidase. Detection of proteins was performed using the ECL detection system.

Immunoprecipitation analysis

One volume of lysate was diluted with 3 volumes of washing buffer [50 m*M* Tris-HCl (pH 7.9), 137 m*M* NaCl, 1 m*M* sodium orthovanadate, 10% (vol/vol) glycerol, and 1% (vol/vol) Nonidet P-40]. Diluted lysates were incubated

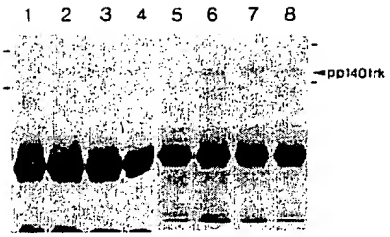


FIG. 1. Tyrosine phosphorylation of p140trk in NGF-treated sympathetic neurons: inhibition by the trk inhibitor K252a. Chick sympathetic neurons (E12) were not treated (lanes 1 and 5) or treated with 10 ng/ml of NGF for 1 (lane 2), 5 (lane 3), 3 (lane 6), 10 (lane 7), or 30 min (lane 8). For lane 4, sympathetic neurons were pretreated with 1 μM K252a for 10 min, followed by incubation with 10 ng/ml of NGF for 1 min. Lanes 1–4 and 5–8 were derived from two independent experiments. Cells were lysed, and trk proteins in the lysate were immunoprecipitated using anti-trk antiserum. Proteins in immunoprecipitates were subjected to SDS-PAGE. After transfer to nitrocellulose, blots were reacted with anti-phosphotyrosine monoclonal antibody. Horizontal upper and lower bars indicate the position of 205- and 116-kDa molecular mass standard proteins, respectively. The arrow on the right points to the position of tyrosine-phosphorylated protein p140trk. The broad bands represent antibody heavy chains in the immunoprecipitate.

with anti-trk antiserum for 4 h, followed by incubation with protein A-Sepharose CL-4B beads for 2 h. After centrifugation the beads were washed three times with washing buffer and heated for 5 min at 95°C in sample buffer according to the procedure of Laemmli (1970). Immunoprecipitated proteins were separated by SDS-PAGE, and immunoblot analysis with anti-phosphotyrosine monoclonal antibody was performed as described before. All experiments were repeated two or three times with similar results.

RESULTS

NGF-stimulated tyrosine phosphorylation of p140trk in cultured chick sympathetic and DRG sensory neurons

We used a combined immunoprecipitation–immunoblot approach to analyze the NGF-stimulated tyrosine phosphorylation of trk in primary neurons. Trk proteins were immunoprecipitated from lysates of NGF-treated sympathetic neurons followed by immunoblot analysis using an anti-phosphotyrosine monoclonal antibody. As can be seen in Fig. 1, tyrosine phosphorylation of p140trk was detectable after stimulation of sympathetic neurons with NGF for 1 min. Treatment with NGF for longer times (up to 30 min) resulted in similar levels of tyrosine phosphorylation of p140trk (see Fig. 1). As expected, NGF-stimulated tyrosine phosphorylation of p140trk could be prevented by preincubation of sympathetic neurons with the trk inhibitor K252a (see Fig. 1).

Similarly, tyrosine phosphorylation of p140trk was observed within 1 min after addition of NGF to chick DRG sensory neurons (Fig. 2). Tyrosine phosphorylation of p140trk could be inhibited by preincubation of DRG sensory neurons with the trk inhibitor K252a (see Fig. 2).

Time-resolved NGF-stimulated tyrosine phosphorylation of a novel protein p105 in cultured chick sympathetic and DRG sensory neurons

We have developed a sensitive method to analyze the tyrosine phosphorylation of proteins in ~50,000 cultured primary neurons. Cells were lysed in a small volume of lysis buffer, and soluble proteins were characterized by immunoblotting using a sensitive and specific monoclonal anti-phosphotyrosine antibody followed by a horseradish peroxidase-conjugated second antibody and an enhanced chemiluminescence detection system.

NGF induced the rapid tyrosine phosphorylation of two proteins in chick sympathetic neurons. A protein with an apparent molecular mass of 105 kDa (p105) became tyrosine-phosphorylated already within 1 min, with phosphorylation peaking at 3 min after exposure to NGF (Fig. 3). On prolonged treatment with NGF, tyrosine phosphorylation of p105 remained stable and declined moderately between 2 and 4 h, the longest interval measured (Fig. 4). Preincubation of sympathetic neurons with the trk inhibitor K252a (see, for review, Knüsel and Hefti, 1992) inhibited the tyrosine phosphorylation of p105 (see Fig. 3). Recombinant NGF stimulated the tyrosine phosphorylation of p105 at the same concentration as did NGF from mouse submaxillary glands (data not shown).

In DRG sensory neurons, NGF induced the weak and transient tyrosine phosphorylation of a protein with an apparent molecular mass of 105 kDa (Fig. 5). Maximal tyrosine phosphorylation of p105 was observed

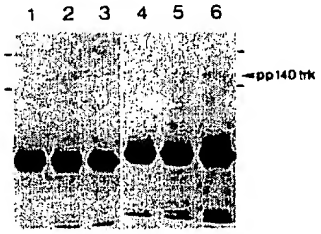


FIG. 2. Tyrosine phosphorylation of p140trk in NGF-treated DRG sensory neurons: inhibition by the trk inhibitor K252a. Chick DRG sensory neurons (E9) were not treated (lanes 1 and 4) or treated with 10 ng/ml of NGF for 1 (lanes 2 and 5) or 5 min (lane 6). For lane 3, DRG sensory neurons were pretreated with 1 μM K252a for 10 min, followed by incubation with 10 ng/ml of NGF for 1 min. Lanes 1–3 and 4–6 were derived from two independent experiments. Cells were lysed, and trk proteins in the lysate were immunoprecipitated using anti-trk antiserum. Proteins in immunoprecipitates were subjected to SDS-PAGE. After transfer to nitrocellulose, blots were reacted with anti-phosphotyrosine monoclonal antibody. Horizontal upper and lower bars indicate the position of 205- and 116-kDa molecular mass standard proteins, respectively. The arrow on the right points to the position of tyrosine-phosphorylated protein p140trk. The broad bands represent antibody heavy chains in the immunoprecipitate.

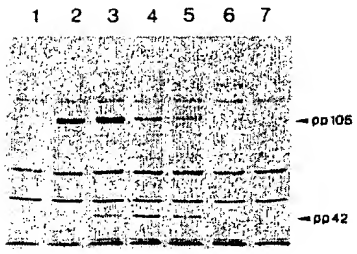


FIG. 3. Tyrosine phosphorylation of proteins in NGF-treated sympathetic neurons: short time course and inhibition by the trk inhibitor K252a. Chick sympathetic neurons (E12) were not treated (lanes 1 and 7) or treated with 10 ng/ml of NGF for 1 (lane 2), 3 (lane 3), 10 (lane 4), or 30 min (lane 5). For lane 6, sympathetic neurons were pretreated with 1 μM K252a for 10 min, followed by incubation with 10 ng/ml of NGF for 10 min. Cells were lysed, and proteins in the lysate were subjected to SDS-PAGE. After transfer to nitrocellulose, blots were reacted with anti-phosphotyrosine monoclonal antibody. Arrows on the right indicate the position of tyrosine-phosphorylated proteins p105 and p42.

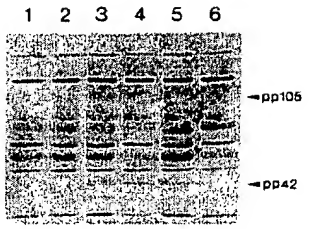


FIG. 5. Tyrosine phosphorylation of proteins in NGF-treated DRG sensory neurons: short time course and inhibition by the trk inhibitor K252a. Chick DRG sensory neurons (E9) were not treated (lane 1) or treated with 10 ng/ml of NGF for 1 min (lane 2), 3 min (lane 3), 10 min (lane 4), or 30 min (lane 5). For lane 6, DRG sensory neurons were pretreated with 1 μM K252a for 10 min, followed by incubation with 10 ng/ml of NGF for 10 min. Cells were lysed, and proteins in the lysate were subjected to SDS-PAGE. After transfer to nitrocellulose, blots were reacted with anti-phosphotyrosine monoclonal antibody. Arrows on the right indicate the position of tyrosine-phosphorylated proteins p105 and p42.

after treatment with NGF for 3 min. In contrast to sympathetic neurons, tyrosine phosphorylation of p105 was hardly detectable after treatment of DRG sensory neurons with NGF for 10 min (see Fig. 5).

Time-resolved NGF-stimulated tyrosine phosphorylation of protein p42 in sympathetic and DRG sensory neurons

After stimulation of sympathetic neurons with NGF for 3 min, we observed the tyrosine phosphorylation of a protein with an apparent molecular mass of 42 kDa (p42) (see Fig. 3). Tyrosine phosphorylation of p42 in sympathetic neurons was maximal after treatment with NGF for 10 min. Between 10 and 30 min after stimulation with NGF the amount of tyrosine-phosphorylated p42 started to decrease to barely detectable levels after 4 h (see Fig. 4). Preincubation of sympathetic neurons with the trk inhibitor K252a prevented the NGF-dependent tyrosine phosphorylation of p42 (see Fig. 3). Recombinant NGF stimulated the tyrosine phosphorylation of p42 at the same concentration as did NGF from mouse submaxillary glands (data not shown).

As in the case with sympathetic neurons, treatment of DRG sensory neurons with NGF for 3 min induced the tyrosine phosphorylation of a protein with a molecular mass of 42 kDa (p42) (see Fig. 5). Tyrosine phosphorylation of p42 was maximal after treatment with NGF for 10 min and remained constant for ~1 h. Longer incubation times of DRG sensory neurons resulted in a decrease in the tyrosine phosphorylation of p42, but the level was still elevated after treatment with NGF for 4 hours (Fig. 6). Preincubation of DRG sensory neurons with the trk kinase inhibitor K252a prevented the NGF-stimulated tyrosine phosphorylation of p42 (see Fig. 5).

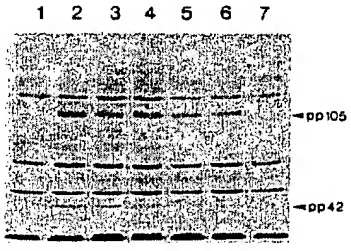


FIG. 4. Tyrosine phosphorylation of proteins in NGF-treated sympathetic neurons: long time course. Chick sympathetic neurons (E12) were not treated (lanes 1 and 7) or treated with 10 ng/ml of NGF for 10 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), or 4 h (lane 6). Cells were lysed, and proteins in the lysate were subjected to SDS-PAGE. After transfer to nitrocellulose, blots were reacted with anti-phosphotyrosine monoclonal antibody. Arrows on the right indicate the position of tyrosine-phosphorylated proteins p105 and p42.

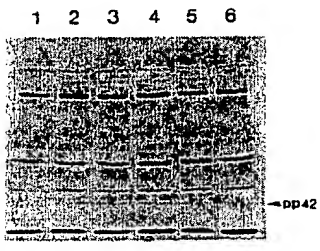


FIG. 6. Tyrosine phosphorylation of proteins in NGF-treated DRG sensory neurons: long time course. Chick DRG sensory neurons (E9) were not treated (lane 1) or treated with 10 ng/ml of NGF for 10 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), or 4 h (lane 6). Cells were lysed, and proteins in the lysate were subjected to SDS-PAGE. After transfer to nitrocellulose, blots were reacted with anti-phosphotyrosine monoclonal antibody. The arrow on the right indicates the position of tyrosine-phosphorylated protein p42.

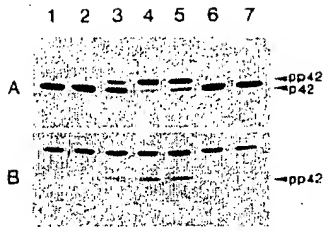


FIG. 7. Identification of pp42 as a tyrosine-phosphorylated MAP kinase in NGF-treated sympathetic neurons. Chick sympathetic neurons (E12) were not treated (lanes 1 and 7) or treated with 10 ng/ml of NGF for 1 (lane 2), 3 (lane 3), 10 (lane 4), or 30 min (lane 5). For lane 6, sympathetic neurons were pretreated with 1 μM K252a for 10 min, followed by incubation with 10 ng/ml of NGF for 10 min. Cells were lysed, and proteins in the lysate were subjected to SDS-PAGE on large low-cross-linker gels and transferred to nitrocellulose. **A:** The blot was incubated with anti-MAP kinase antiserum. **B:** After removal of bound antibodies the blot was reincubated with anti-phosphotyrosine monoclonal antibody. Arrows on the right indicate the position of shifted tyrosine-phosphorylated MAP kinase p42 (pp42) and the position of unshifted unphosphorylated MAP kinase p42 (p42).

Identification of p42 as a MAP kinase

Molecular mass and time kinetics of tyrosine phosphorylation of p42 in NGF-treated sympathetic neurons led us to the suggestion that p42 might belong to the family of MAP kinases. Further evidence for this hypothesis was obtained by the fact that, after removal of anti-phosphotyrosine monoclonal antibody from the immunoblot, reprobing with an antiserum directed against the MAP kinases extracellular signal-regulated kinase (ERK) 1 and ERK2 revealed a band with a molecular mass of 42 kDa (data not shown). To prove that p42 indeed belongs to the family of MAP kinases, we used a gel shift assay that discriminates between phosphorylated and unphosphorylated forms of the protein.

Proteins in lysates from untreated and NGF-treated chick sympathetic neurons were blotted from large SDS-polyacrylamide gels with low cross-linker, and blots were incubated with anti-MAPK antiserum. As can be seen in Fig. 7A, this antiserum detected a single protein species in untreated cells. Stimulation of sympathetic neurons with NGF for 3 min resulted in a shift of part of the MAP kinase to a higher apparent molecular mass (see Fig. 7A). Stimulation with NGF for 10 min was accompanied by a shift of most of the MAP kinase, whereas treatment with NGF for 30 min led to an increase in the amount of the unshifted form of MAP kinase. Preincubation of NGF-stimulated sympathetic neurons with K252a resulted in the appearance of the unshifted form of MAP kinase (see Fig. 7A).

To verify that the shifted species of MAP kinase was indeed tyrosine-phosphorylated, the blot was stripped and reincubated with anti-phosphotyrosine monoclonal antibody. As can be seen in Fig. 7B, only the shifted form of MAP kinase was stained with anti-phosphotyrosine monoclonal antibody, demonstrating that NGF treatment of sympathetic neurons resulted in a tyrosine-phosphorylated form of MAP kinase.

Other tyrosine-phosphorylated proteins

Several other tyrosine-phosphorylated proteins could be detected in unstimulated sympathetic and DRG sensory neurons. As expected, tyrosine phosphorylation of these proteins was not prevented by preincubation of neurons with the trk inhibitor K252a (see Figs. 3 and 5). Preincubation of anti-phosphotyrosine monoclonal antibody with phosphotyrosine inhibited the binding of the antibody to both basally and NGF-stimulated tyrosine-phosphorylated proteins in sympathetic and DRG sensory neurons (data not shown).

DISCUSSION

Although sympathetic and DRG sensory neurons derived from the chick PNS represent "classical" NGF-responsive systems, only limited information is available on the mechanism of NGF-mediated signal transduction beyond the trk receptor activation (Borasio et al., 1993; Clary et al., 1994). Specifically, the intracellular signaling cascade leading to neuronal survival is virtually unknown. As we have demonstrated previously, DRG sensory neurons are absolutely dependent on p21ras activity: Intracellular application of activated p21ras replaces the NGF requirement for survival (Borasio et al., 1989); and, conversely, intracellular application of function-blocking anti-p21ras Fab fragments inhibits the NGF signal pathway for survival (Borasio et al., 1993). Consistently, activation of p21ras by NGF has been shown recently in chick DRG sensory neurons (Ng and Shooter, 1993). However, no effect of activated p21ras was found in chick sympathetic neurons, and, correspondingly, the anti-p21ras Fab fragments could influence neither neurite outgrowth nor survival induced by NGF (Borasio et al., 1993). Accordingly, we present evidence here that the time-resolved tyrosine phosphorylation pattern of proteins is distinctly different between both neural crest-derived neuronal subtypes at a branchpoint downstream the p140trk receptor.

The action of NGF initiated by the aggregation of the p140trk receptor is followed by rapid cross-phosphorylation at defined tyrosine residues of the intracellular domain (Obermeier et al., 1994; Stephens et al., 1994). Here, maximal tyrosine phosphorylation of p140trk can be observed after treatment of sympathetic neurons with NGF for 1 min. In sympathetic neurons tyrosine phosphorylation persisted for up to 30 min, the longest interval measured. Consistently, it has been shown recently that persistent trkA receptor aggregation by antibodies against the extracellular portion of trkA is sufficient to cause its autophosphorylation leading to neurite outgrowth and survival (Clary et al., 1994). Moreover, in trkA tyrosine kinase mouse gene knock-out mutants the sympathetic ganglia were strik-

ingly affected (Smeyne et al., 1994), demonstrating that the tyrosine kinase activity of trkA is essential for survival.

Downstream to the activation of p140trk receptor, NGF induces the rapid and persistent tyrosine phosphorylation of an as yet uncharacterized protein, p105, selectively in chick sympathetic neurons. Tyrosine phosphorylation of p105 can be detected already 30 s after treatment with NGF (data not shown), indicating that this is an early step of NGF-induced signal transduction. However, activated p140trk and tyrosine-phosphorylated p105 may probably not interact directly because pp105 is not coprecipitated when using anti-trk antibodies (see Fig. 1).

Tyrosine-phosphorylated p105 undergoes a time-dependent progressive shift to higher apparent molecular masses after treatment of sympathetic neurons with NGF. Blots from enlarged SDS-polyacrylamide gels using low cross-linker indicate the presence of multiple species of pp105 (data not shown). Most likely, tyrosine-phosphorylated p105 is subjected to additional posttranslational modifications.

In contrast to sympathetic neurons, NGF-stimulated tyrosine phosphorylation of p105 is transient in DRG sensory neurons, showing a profound difference between both subtypes of neurons. As has been demonstrated previously, NGF induces the activation of p21ras in DRG sensory neurons (Ng and Shooter, 1993). The activation of p21ras has been shown in many systems to initiate a cascade of protein phosphorylation events, leading finally to the activation of MAP kinases. Consistent with this idea, NGF stimulates the rapid tyrosine phosphorylation of a single form of MAP kinase (p42) in both chick sympathetic and DRG sensory neurons. We have identified this protein as a MAP kinase using an anti-MAP kinase antiserum directed against the mammalian ERK1 and ERK2 proteins.

Unlike NGF-stimulated tyrosine phosphorylation of p105, similarities exist between sympathetic and DRG sensory neurons with respect to the onset of tyrosine phosphorylation of MAP kinase p42. Whereas in both subtypes of neurons tyrosine phosphorylation of MAP kinase p42 is induced after treatment with NGF for 3 min, the decrease in tyrosine phosphorylation is differentially regulated in these systems. In sympathetic neurons maximal tyrosine phosphorylation of MAP kinase p42 can be observed after treatment with NGF for 10–30 min, whereas in DRG sensory neurons maximal tyrosine phosphorylation of MAP kinase p42 is more long-lasting and decreases after stimulation with NGF for 1 h. The difference in the time course of NGF-stimulated tyrosine phosphorylation of MAP kinase p42 between sympathetic and DRG sensory neurons may be explained by either a different strength of signal input or different inactivation of tyrosine-phosphorylated MAP kinase p42 by tyrosine phosphatase(s) (Nebreda, 1994).

Using the gel shift assay for detection of phosphorylated proteins, we observed that the tyrosine-phosphorylated form of MAP kinase p42 shifts to a higher apparent molecular mass after treatment of sympathetic neurons with NGF. Because it has been shown that the more slowly migrating form of MAP kinase ERK2 corresponds to the activated phosphorylated species (Leevers and Marshall, 1992), we can estimate from our blots the percentage of MAP kinase that becomes activated by NGF. Treatment of sympathetic neurons for 10 min with 10 ng/ml of NGF activates ~90% of the MAP kinase p42, whereas only a smaller fraction of MAP kinase is activated after stimulation with NGF for 3 or 30 min.

As was recently shown, not only the function of p21ras but also the activity of MEK1 is essential for NGF-stimulated neurite outgrowth in PC12 cells (Cowley et al., 1994). However, because in sympathetic neurons p21ras activity does not influence survival (Borasio et al., 1993), its downstream effects are not necessarily coupled to prevent apoptosis. Thus, it is not surprising that the time course of NGF-stimulated tyrosine phosphorylation of MAP kinase p42 is different between chick DRG sensory and sympathetic neurons. It is unclear at the moment whether MAP kinase p42 is activated via a p21ras-independent pathway in chick sympathetic neurons. As was shown earlier, activation of p21ras is necessary for NGF-stimulated survival and neurite outgrowth of chick DRG sensory neurons but not sympathetic neurons. Our data provide evidence that in DRG sensory neurons NGF signal transduction proceeds via the activation of p21ras and subsequently MAP kinase p42.

In addition to the neurotrophins NGF and neurotrophin-3, survival of chick sympathetic neurons can be induced by treatment with ciliary neurotrophic factor (Barbin et al., 1984; Ernsberger et al., 1989), a high K^+ concentration (Wakade et al., 1983), or forskolin (Wakade et al., 1990). It is interesting that treatment with these agents does not stimulate the tyrosine phosphorylation of p105, whereas neurotrophin-3 does so (data not shown). Our results support the working hypothesis that variant signal pathways may exist for survival in sympathetic neurons and suggest that tyrosine phosphorylation of p105 is only involved in stimulation of survival via the neurotrophins NGF and neurotrophin-3.

Rat pheochromocytoma PC12 cells (Greene and Tischler, 1976) are commonly used as a model system for sympathetic neurons. Our study indicates that several differences exist in NGF-stimulated tyrosine phosphorylation of proteins between chick sympathetic neurons and transformed PC12 cells:

a. In chick sympathetic neurons maximal tyrosine phosphorylation of p140trk can be observed within 1 min after treatment with NGF and persists for up to 30 min. This time course is different from that observed for PC12 cells, where maximal tyrosine phosphorylation of p140trk was reported to occur after

treatment with NGF for 5 min and decreased thereafter (Kaplan et al., 1991).

b. In contrast to PC12 cells, where NGF induces the tyrosine phosphorylation of many proteins (Maher, 1988; Miyasaka et al., 1991; authors' unpublished data), NGF stimulates the rapid and reproducible tyrosine phosphorylation of two proteins in chick sympathetic and DRG sensory neurons. The observed difference in the number of tyrosine-phosphorylated proteins between NGF-treated primary neurons and PC12 cells may be due to the fact that PC12 cells are transformed and that these proteins may therefore be involved in signal transduction leading to mitosis.

c. In contrast to PC12 cells, where three types of MAP kinase (p43erk1, p41erk2, and p45erk4) become tyrosine-phosphorylated after treatment with NGF (Boulton et al., 1991; Ahn et al., 1992; Lloyd and Wooten, 1992), NGF stimulates the rapid tyrosine phosphorylation of a single form of MAP kinase (p42) in chick sympathetic and DRG sensory neurons.

Our results provide evidence that NGF signal transduction in primary neurons and transformed cells of neuronal origin is different at several steps. Because transformation and indefinite division of PC12 cells are in contrast to characteristics of postmitotic neurons, experiments with primary neurons may result in more valuable information regarding NGF signal transduction.

In conclusion, determination of the time-resolved protein tyrosine phosphorylation pattern shows that the signaling pathways of NGF diverge downstream the trk receptor level between sympathetic and DRG sensory neurons of the chick. We have used the chick sympathetic neurons as a model to establish p21ras-independent neurotrophin signaling pathways. The rapid and persistent tyrosine phosphorylation of a protein p105 with unknown biochemical function may imply that it plays an important role in this p21ras-independent NGF signaling pathway.

Acknowledgment: We thank D. Peter for expert technical assistance and K. Grabert for photographic work. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

REFERENCES

Ahn N. G., Robbins D. J., Haycock J. W., Seger R., Cobb M. H., and Krebs E. G. (1992) Identification of an activator of the microtubule-associated protein 2 kinases ERK1 and ERK2 in PC12 cells stimulated with nerve growth factor or bradykinin. *J. Neurochem.* **59,** 147–156.

Barbacid M. (1993) Nerve growth factor: a tale of two receptors. *Oncogene* **8,** 2033–2042.

Barbin G., Manthorpe M., and Varon S. (1984) Purification of the chick eye ciliary neuronotrophic factor. *J. Neurochem.* **43,** 1468–1478.

Barker P. A. and Shooter E. M. (1994) Disruption of NGF binding to the low affinity neurotrophin receptor $p75^{LNTR}$ reduces NGF binding to trkA on PC12 cells. *Neuron* **13,** 203–215.

Basu T., Warne P. H., and Downward J. (1994) Role of Shc in the activation of Ras in response to epidermal growth factor and nerve growth factor. *Oncogene* **9,** 3483–3491.

Boguski M. S. and McCormick F. (1993) Proteins regulating Ras and its relatives. *Nature* **366,** 643–654.

Borasio G. D., John J., Wittinghofer A., Barde Y.-A., Sendtner M., and Heumann R. (1989) ras p21 protein promotes survival and fiber outgrowth of cultured embryonic neurons. *Neuron* **2,** 1087–1096.

Borasio G. D., Markus A., Wittinghofer A., Barde Y.-A., and Heumann R. (1993) Involvement of ras p21 in neurotrophin-induced response of sensory, but not sympathetic neurons. *J. Cell Biol.* **121,** 665–672.

Boulton T. G., Nye S. H., Robbins D. J., Ip N. Y., Radziejewska E., Morgenbesser S. D., DePinho R. A., Panayotatos N., Cobb M. H., and Yancopoulos G. D. (1991) ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65,** 663–675.

Chao M. V. (1992) Neurotrophin receptors: a window into neuronal differentiation. *Neuron* **9,** 583–593.

Clary D. O., Weskamp G., Austin L. R., and Reichardt L. F. (1994) trkA crosslinking mimics neuronal responses to nerve growth factor. *Mol. Biol. Cell* **5,** 549–563.

Cowley S., Paterson H., Kemp P., and Marshall C. J. (1994) Activation of MAP kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77,** 841–852.

Dobrowsky R. T., Werner M. H., Castellino A. M., Chao M. V., and Hannun Y. A. (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. *Science* **265,** 1596–1599.

Ernsberger U., Sendtner M., and Rohrer H. (1989) Proliferation and differentiation of embryonic chick sympathetic neurons: effects of ciliary neurotrophic factor. *Neuron* **2,** 1275–1284.

Greene L. A. and Tischler A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73,** 2424–2428.

Hagag N., Halegoua S., and Viola M. (1986) Inhibition of growth factor-induced differentiation of PC12 cells by microinjection of antibody to ras p21. *Nature* **319,** 680–682.

Hashimoto Y., Matuoka K., Takenawa T., Muroya K., Hattori S., and Nakamura S. (1993) Different interactions of Grb2/Ash molecule with the NGF and EGF receptors in rat pheochromocytoma PC12 cells. *Oncogene* **9,** 869–875.

Heumann R. (1994) Neurotrophin signalling. *Curr. Opin. Neurobiol.* **4,** 668–679.

Jaiswal R. K., Moodie S. A., Wolfman A., and Landreth G. E. (1994) The mitogen-activated protein kinase cascade is activated by B-raf in response to nerve growth factor through interaction with $p21^{ras}$. *Mol. Cell. Biol.* **14,** 6944–6953.

Jing S., Tapley P., and Barbacid M. (1992) Nerve growth factor mediates signal transduction through trk homodimer receptors. *Neuron* **9,** 1067–1079.

Kaplan D. R., Martin-Zanca D., and Parada L. F. (1991) Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature* **350,** 158–160.

Knüsel B. and Hefti F. (1992) K-252 compounds: modulators of neurotrophin signal transduction. *J. Neurochem.* **59,** 1987–1996.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lange-Carter C. A. and Johnson G. L. (1994) Ras-dependent growth factor regulation of MEK kinase in PC12 cells. *Science* **265,** 1458–1461.

Leevers S. J. and Marshall C. J. (1992) Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoprotein. *EMBO J.* **11,** 569–574.

Lindsay R. M., Thoenen H., and Barde Y.-A. (1985) Placode and neural crest-derived sensory neurons are responsive at early

developmental stages to brain-derived neurotrophic factor. *Dev. Biol.* **112,** 319–328.

Lloyd E. D. and Wooten M. W. (1992) $^{p42/44}$MAP kinase is a component of the neurogenic pathway utilized by nerve growth factor in PC12 cells. *J. Neurochem.* **59,** 1099–1109.

Maher P. A. (1988) Nerve growth factor induces protein-tyrosine phosphorylation. *Proc. Natl. Acad. Sci. USA* **85,** 6788–6791.

Meakin S. O. and Shooter E. M. (1992) The nerve growth factor family of receptors. *Trends Neurosci.* **15,** 323–331.

Miyasaka T., Stemberg D. W., Miyasaka J., Sherline P., and Saltiel A. R. (1991) Nerve growth factor stimulates tyrosine phosphorylation in PC-12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA* **88,** 2653–2657.

Nakamura K., Tanaka T., Kuwahara A., and Takeo K. (1985) Microassay for proteins on nitrocellulose filter using protein dye-staining procedure. *Anal. Biochem.* **148,** 311–319.

Nebreda A. R. (1994) Inactivation of MAP kinases. *Trends Biochem. Sci.* **19,** 1–2.

Ng F. L. and Shooter E. M. (1993) Activation of p21^{ras} by nerve growth factor in embryonic sensory neurons and PC12 cells. *J. Biol. Chem.* **268,** 25329–25333.

Obermeier A., Bradshaw R. A., Seedorf K., Choidas A., Schlessinger J., and Ullrich A. (1994) Neuronal differentiation signals are controlled by nerve growth factor receptor/Trk binding sites for SHC and PLCγ. *EMBO J.* **13,** 1585–1590.

Ohmichi M., Decker S. J., and Saltiel A. R. (1992*a*) Activation of phosphatidylinositol-3 kinase by nerve growth factor involves indirect coupling of the *trk* proto-oncogene with src homology 2 domains. *Neuron* **9,** 769–777.

Ohmichi M., Pang L., Decker S. J., and Saltiel A. R. (1992*b*) Nerve growth factor stimulates the activities of the raf-1 and the mitogen-activated protein kinases via the *trk* protooncogene. *J. Biol. Chem.* **267,** 14604–14610.

Ohmichi M., Matuoka K., Takenawa T., and Saltiel A. R. (1994) Growth factors differentially stimulate the phosphorylation of Shc proteins and their association with Grb2 in PC-12 pheochromocytoma cells. *J. Biol. Chem.* **269,** 1143–1148.

Oshima M., Sithanandam G., Rapp U. R., and Guroff G. (1991) The phosphorylation and activation of B-raf in PC12 cells stimulated by nerve growth factor. *J. Biol. Chem.* **266,** 23753–23760.

Qiu M.-S. and Green S. H. (1992) PC12 cell neuronal differentiation is associated with prolonged p21ras activity and consequent prolonged ERK activity. *Neuron* **9,** 705–717.

Saltiel A. R. and Decker S. J. (1994) Cellular mechanisms of signal transduction for neurotrophins. *Bioessays* **16,** 405–411.

Smeyne R. J., Klein R., Schnapp A., Long L. K., Bryant S., Lewin A., Lira S. A., and Barbacid M. (1994) Severe sensory and sympathetic neuropathies in mice carrying a disrupted trk/NGF receptor gene. *Nature* **368,** 246–249.

Snider W. D. (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell* **77,** 627–638.

Soltoff S. P., Rabin S. L., Cantley L. C., and Kaplan D. R. (1992) Nerve growth factor promotes the activation of phosphatidylinositol 3-kinase and its association with the trk tyrosine kinase. *J. Biol. Chem.* **267,** 17472–17477.

Stephens R. M., Loeb D. M., Copeland T. D., Pawson T., Greene L. A., and Kaplan D. R. (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-γ1 to mediate NGF responses. *Neuron* **12,** 691–705.

Suda K., Barde Y. A., and Thoenen H. (1978) Nerve growth factor in mouse and rat serum: correlation between bioassay and radioimmunoassay determination. *Proc. Natl. Acad. Sci. USA* **75,** 4042–4046.

Szeberenyi J., Cai H., and Cooper G. M. (1990) Effect of a dominant inhibitory Ha-*ras* mutation on neuronal differentiation of PC12 cells. *Mol. Cell. Biol.* **10,** 5324–5332.

Thomas S. M., DeMarco M., D'Arcangelo G., Halegoua S., and Brugge J. S. (1992) Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell* **68,** 1031–1040.

Vaillancourt R. R., Gardner A. M., and Johnson G. L. (1994) B-Raf-dependent regulation of the MEK-1/mitogen-activated protein kinase pathway in PC12 cells and regulation by cAMP. *Mol. Cell. Biol.* **14,** 6522–6530.

Vetter M. L., Martin-Zanca D., Parada L. F., Bishop J. M., and Kaplan D. R. (1991) Nerve growth factor stimulates tyrosine phosphorylation of phospholipase C-γ1 by a kinase activity associated with the product of the *trk* protooncogene. *Proc. Natl. Acad. Sci. USA* **88,** 5650–5654.

Wakade A. R., Edgar D., and Thoenen H. (1983) Both nerve growth factor and high K^+ concentrations support the survival of chick embryo sympathetic neurons: evidence for a common mechanism of action. *Exp. Cell Res.* **144,** 377–384.

Wakade A. R., Bhave S. V., Malhotra R. K., and Wakade T. D. (1990) Forskolin mediates the survival of nerve growth factor-dependent sympathetic neurons of chick embryo by a cyclic AMP-independent mechanism. *J. Neurochem.* **54,** 1281–1287.

Wood K. W., Sarnecki C., Roberts T. M., and Blenis J. (1992) ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, raf-1, and RSK. *Cell* **68,** 1041–1050.

Journal of Neurocytology 15, 789–798 (1986)

Comparative dynamics of retrograde transport of nerve growth factor and horseradish peroxidase in rat lumbar dorsal root ganglia

HENRY K. YIP and EUGENE M. JOHNSON, JR

Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110, USA

Received 14 May 1986; accepted 17 June 1986

Summary

The dynamics of the retrograde transport of [^{125}I] nerve growth factor (NGF) and horseradish peroxidase (HRP) in dorsal root ganglion (DRG) neurons were studied in rats. After injection of [^{125}I]NGF or HRP into crushed sciatic nerve, labelling was examined in spinal nerves, dorsal root ganglia, dorsal roots and spinal cord. Retrograde transport of either [^{125}I]NGF or HRP was first observed in DRG neurons 6 h after injection: The maximal rate of transport (7 mm h^{-1}) was similar for both proteins. Significant differences in the sizes of DRG neurons labelled by [^{125}I]NGF were observed and were dependent upon survival time. No such difference was seen in HRP-injected animals. At 6 h after injection, 60% of all the HRP-labelled cells had a diameter of more than 25 μm, whereas 90% of all the [^{125}I]NGF-labelled neurons had a diameter of less than 25 μm. With increasing survival times there was a gradual shift in the size of [^{125}I]NGF-labelled neurons towards larger diameters. Thus, 24 h after the [^{125}I]NGF injection, 83% of the labelled cells had a diameter greater than 25 μm. The data suggest that small diameter neurons retrogradely transport and turnover NGF faster than larger diameter neurons. There was a preferential accumulation of silver grains in small DRG neurons (mean diameter 25 μm) at early survival times (4 and 8 h); at the later survival time (24 h) the reverse was observed, i.e. larger neurons (mean diameter 42 μm) were labelled. In contrast, the mean diameter of HRP-labelled neurons remained constant (30 μm) at all times after injection. The total number of neurons ultimately labelled (~ 80–85%) appeared to be the same with both tracers. In addition, the lack of transganglionic transport of NGF into the spinal cord and the short time span of the observable accumulated radioactivity in DRG neurons suggest the rate of degradation of transported NGF seems to be faster than HRP. As a practical matter, these data indicate that observing cells within DRG which accumulate retrogradely transported [^{125}I]NGF at any one time gives an inaccurate picture of the size properties of cells capable of transporting the ligand.

Introduction

Neurons take up macromolecular substances at the nerve terminals and transport then intra-axonally in a retrograde direction (i.e. towards the cell body). Certain toxins and viruses were among the first molecules or particles for which such retrograde transport was demonstrated (Kristensson, 1975). The retrograde transport of horseradish peroxidase (HRP) has been an invaluable tool for tracing neuronal pathways in both central and peripheral nervous systems (Nauta *et al.*, 1974; LaVail, 1975). However, the physiological importance and mechanism of the retrograde transport of these exogenous macromolecular substances remain uncertain. It is a mechanism whereby the terminals of nerve fibres may sample and transport information from their local surroundings back to the perikarya. Thus, a retrograde axonal transport system fulfils the regulatory role of conveying chemical messages from end organs back to the innervating nerve cells (Cragg, 1970; Hendry & Iversen, 1973; Hamburger, 1975). Studies performed during the last decade with radiolabelled nerve growth factor (NGF) have provided strong evidence in favour of this hypothesis and, at the same time, have indicated that NGF is a trophic factor released by the target organs to be taken up selectively by the sympathetic and sensory nerve endings (see review by Thoenen & Barde, 1980).

The selectivity of the uptake and retrograde axonal transport of labelled NGF, in contrast to HRP, depends on the binding of NGF to specific receptors on the surface of the nerve terminals (Hendry, 1975; Stöckel *et al.*, 1975). Other protein molecules which share many similar physiochemical properties with NGF, but do not display any biological function on the sympathetic and sensory neurons, are not

0300-4864/86 $03.00 + .12

retrogradely transported to the cell bodies. The high selectivity and specificity of the NGF binding sites can also be demonstrated by the fact that a minor chemical modification of NGF molecules results in both a loss of biological activity and of NGF retrograde transport (Stöckel *et al.*, 1974). Moreover, not all macromolecules are transported in all neurons; for example, HRP is taken up and transported by almost all neurons, while NGF is taken up by sympathetic and sensory dorsal root ganglion (DRG) neurons but not by motor neurons.

It has been previously reported that, after injection of [^{125}I]NGF into the rat forepaw, the accumulation of retrogradely transported NGF in sensory neurons is observed in the population of large neurons (> 25 μm) in the DRG (Stöckel *et al.*, 1975). Since there is a heterogeneous population of neurons in the mammalian DRG which can be distinguished from each other by morphological (Parfianowicz *et al.*, 1971), histochemical (Kalina & Wolman, 1970) and biochemical (Harmar & Keen, 1981) characteristics, and since the small neurons (< 25 μm) have been shown to be responsive to NGF (Kessler & Black, 1980; Kornblum & Johnson, 1982) and to NGF deprivation (Johnson *et al.*, 1980), the failure to demonstrate retrograde transport of [^{125}I]NGF by small neurons is puzzling.

One of the objectives of the present study was to determine what population of DRG neurons retrogradely transport [^{125}I]NGF by comparing the number of neurons accumulating radiolabelled NGF to the number accumulating HRP injected similarly. In addition, we wished to determine if there were any differences in the time course of appearance or accumulation of [^{125}I]NGF or HRP within the heterogeneous populations of neurons in the sensory ganglia. This was accomplished by injecting [^{125}I]NGF or HRP into a crush site in the sciatic nerve. Previously, we have found that 85% of the neurons in the L5 DRG were retrogradely labelled by this protocol (Yip *et al.*, 1984) and that efficient receptor-mediated retrograde transport of [^{125}I]NGF was also observed from such a crush site (Yip & Johnson, 1983). The results show that all DRG neurons retrogradely transport [^{125}I]NGF; surprisingly, and in contrast to HRP, different sizes of DRG neurons have different time courses of appearance and accumulation of [^{125}I]NGF.

Methods and materials

PREPARATION OF [^{125}I]NGF

The 2.5S mouse NGF was prepared from submaxillary glands of adult male mice as described by Bocchini & Angeletti (1969). For radioiodination (Marchalonis, 1969), a mixture of NGF (10 μg), Na^{125}I (1 mCi, Amersham), hydrogen peroxide (0.08 M) and phosphate buffer (0.2 M, pH 6.8) to a total volume of 50 μl was incubated for 15 min at room temperature. A total of 150 μl of phosphate buffer (0.05 M, pH 7.5) with bovine serum albumin (0.2%) and protamine sulphate (0.1%) was then added and the mixture was dialysed overnight against two changes of 0.1 M phosphate buffer (pH 7.4). The efficiency of labelling was 70–90%; after dialysis, incorporation of greater than 96% was determined by thin layer chromatography. Final concentration of NGF was 25 ng μl^{-1} with specific activities of ~ 80 μCi μg^{-1}. Bioactivity of [^{125}I]NGF was verified by the accumulation of radioactivity in the ipsilateral superior cervical ganglia (SCG) of the rat after injection of 2 μl of radiolabelled NGF into the anterior eye chamber. Iodinated NGF was used within a week of preparation.

INJECTIONS

Sprague–Dawley rats weighing 250–300 g (Chappel, St. Louis) were anaesthetized with chloral hydrate (350 mg per 1000 g body weight). The sciatic nerve was exposed in midthigh and crushed with jeweller's forceps at the tendon of *obturator internus*. A total of 2 μl of [^{125}I]NGF or 30% aqueous HRP (Sigma VI) solution was injected into the crush site through a glass micropipette connected to a 10 μl Hamilton syringe.

DETERMINATION OF RADIOACTIVITY

Between 4 and 96 h after injection of [^{125}I]NGF, rats were anaesthetized and perfused with 10% formalin in phosphate buffer. The L5 DRG were dissected out and their gamma emission was determined in a Beckman γ-counter.

AUTORADIOGRAPHY

L5 DRG with attached stumps of spinal nerves, as well as dorsal roots and corresponding spinal cord segments, were postfixed, dehydrated and embedded in paraffin. Serial sections, 8 μm thick, were dewaxed and dipped in Kodak NTB-2 emulsion, then stored in desiccated, lightproof boxes for a week. The slides were developed with Kodak D-19 solution, stained with toluidine blue and mounted in Permount. Cells with more than seven grains (background level) over the cytoplasm were designated as labelled.

HRP HISTOCHEMISTRY

The rats were sacrificed after various survival times and fixed by transcardial perfusion with a mixture of 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.4. Samples of L5 DRG with the attached spinal nerves and dorsal roots, as well as the L5 segments of the spinal cord, were postfixed in the same fixative for several hours, transferred into 0.1 M sodium phosphate buffer containing 30% sucrose and stored overnight at 4°C. Serial sections, 30 μm thick, were cut on a freezing microtome in longitudinal or transverse planes. HRP histochemistry was performed by using the tetramethylbenzidine procedure as described by Mesulam (1978). Sections were counterstained with 1% Neutral Red.

MORPHOMETRIC ANALYSIS

Cell counting

Cell number and size were determined from coded slides of three or four animals for each of the five time points studied (6, 8, 16, 24 and 96 h). The sample consisted of three separate histological sections, approximately five to seven sections apart, which were chosen to represent the maximum longitudinal sections of each ganglion. These sections were examined in the light microscope using brightfield and/or darkfield illumination for the presence of HRP-labelled or [^{125}I]NGF-labelled cells. These were counted at a magnification of × 200, no correction being made for double counting except for the omission of obvious offcuts (profiles < 5–6 μm in diameter). This might have resulted in slight over-estimation of total cell numbers, although, as most sections (longitudinal) were aligned with the shortest axis of the majority of cells, the error would be expected to be small (< 10%). Both the HRP-labelled or [^{125}I]NGF-labelled neurons and the remaining non-labelled neurons in the L5 DRG were counted. A minimum of 1000–1500 cells were counted in each ganglion for each injection time.

Cell size

Measurements of cell size were made on the same sections as the neuronal counts. Size measurements were made on all cell profiles that contained nuclei. Two methods were used to measure perikaryal diameters. For the initial screening (see Figs 2, 3), measurements were made through a light microscope equipped with an eyepiece micrometer at a magnification of × 200. The cell diameter was calculated as the mean of the longest axis and the axis perpendicular to it at its middle. Since sensory neurons within the DRG have been divided into two classes on the basis of perikaryal size (Lieberman, 1976; Lawson, 1979; Lawson & Nickels, 1980), neurons of less than 25 μm in diameter were defined as small cells and those with a diameter greater than 25 μm were defined as large cells. For more detailed analysis of cell size distribution (see Fig. 4), frequency histograms of mean diameters were generated by tracing the perikarya of all labelled neurons onto a sheet of paper with the use of a Zeiss Camera lucida attached to the microscope (final magnification × 470). The drawings of neuronal perikarya were then traced with a stylus onto a Summagraphics Tablet and entered into a PDP 11/44 computer. With computer analysis the cell size histograms were generated for each section, each ganglion and the entire neuronal sample studied (Yip *et al.*, 1984).

Results

Retrograde transport of [^{125}I]NGF in L5 DRG

The time course of accumulation of radioactivity in L5 DRG after injection of [^{125}I]NGF at the crush site is shown in Fig. 1A. No radioactivity was seen at 4 h, but then radioactivity appeared rapidly, reaching a maximum at 8 h. This time course is the same as we have seen in previous studies (Yip & Johnson, 1983). Radioactivity declined rapidly thereafter with a 90% reduction by 96 h. Light microscopic autoradiography of the L5 DRG of the injected side revealed that the increase in the total percentage of cells in the ganglion which were labelled correlated well with the increase in the radioactivity accumulated in the ganglia (Fig. 1B). No labelling (above background)

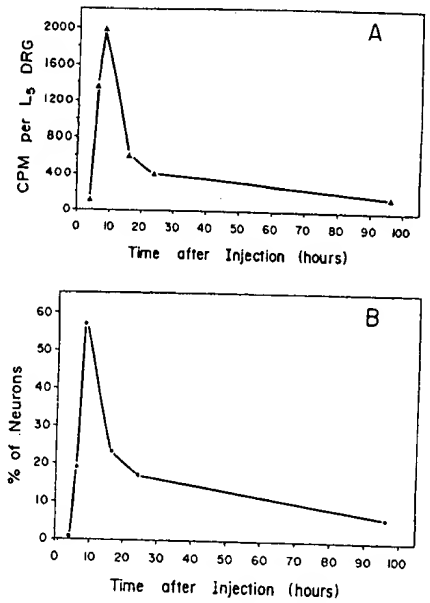


Fig. 1. Correlation between the amount of radioactivity accumulated in L5 DRG after injection of [^{125}I]NGF into a crushed sciatic nerve and the percentage of labelled cells in the same ganglion. (A) Amounts of radioactivity accumulated at various times after injections. (B) Percentage of labelled neurons at corresponding time points.

had accumulated in the neuronal cell bodies of the ipsilateral DRG at 4 h after injection of [^{125}I]NGF into the crushed sciatic nerve. After 6 h, however, 28% of the cells in L5 DRG contained labelling. The majority (57%) of the neurons were labelled after 8 h. The percentage of labelled cells dropped sharply after 16 h and continued to decline through 24 h (Fig. 2A). By 96 h after injection, there was little labelling in L5 DRG.

A gradient of labelling in terms of neuronal size is observed over the 6 to 96 h time course. Examples of [^{125}I]NGF-labelled neurons of different sizes after 6 and 24 h are shown in Fig. 3. Small neurons are labelled first, followed progressively by larger neurons (Figs 2B, 3, 4A). Fig. 2A shows that radioactively labelled small neurons (< 25 μm) occur primarily after 6 and 8 h, with a maximum after 8 h followed by a sharp decline after 16 h and beyond. Heavy labelling of small cells (indicated by S) 6 h after the injection of [^{125}I]NGF is shown in Fig. 3A and B, while absence of labelling of large cells (indicated by L) is also seen. Concurrent with the decline of

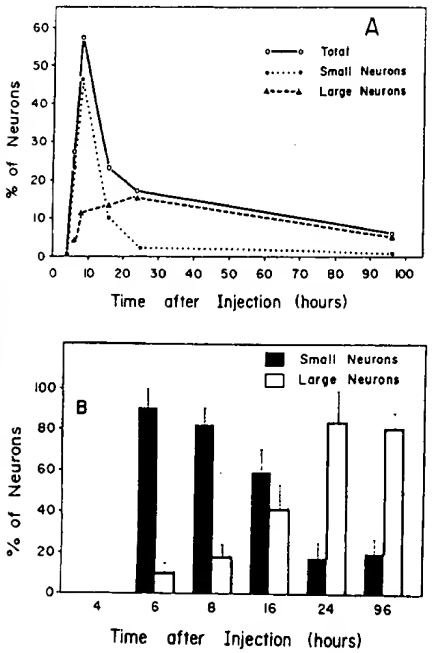


Fig. 2. Percentage of labelled neurons in L5 DRG at various times after injection of [^{125}I]NGF into a crushed sciatic nerve. (A) Percentage of labelled cells in the total population of neurons in L5 DRG. (B) Comparison between the percentage of labelled cells which were small (< 25 μm) or large (> 25 μm) at various times. Note a significant amount of small neurons are labelled at earlier time points than at later time points. The reverse is true for the large, labelled neurons.

heavily labelled small neurons after 8 h, an increase in both the intensity and percentage of labelling of large neurons occurs in the period between 8 and 24 h (Figs 2B, 3C, D, 4). There is, therefore, minimal overlap in the labelling of small and large cells in both early (6 and 8 h) and late (24 and 96 h) time points. At 16 h, similar percentages of both small (59%) and large (41%) neurons are labelled with [^{125}I]NGF (Fig. 2B).

The size distribution of the entire population of neurons was determined by a computer-assisted quantitative analysis of between 200 and 700 labelled neurons from three or four animals. The mean diameter of all [^{125}I]NGF-labelled cells after 6 and 24 h are plotted in 4-μm bins against frequency (Fig. 4A). At 6 h after [^{125}I]NGF injection there is a continuum of cell sizes extending over a range from 9 to 44 μm, with a mean diameter of 22 μm (Fig. 4). At 24 h after the injection, the size frequency spectrum is much broader with an upper boundary of cell size distribution of 48 μm and a mean diameter of [^{125}I]NGF-labelled neurons of 32 μm (Fig. 4). Examination of individual histograms at each time point studied (6, 8, 16, 24 and 96 h) demonstrated a gradual shift toward larger sizes (data not shown). There is also a progressive labelling shift from smaller to larger neurons labelled up to 24 h shown by plotting the mean neuronal size as a function of time after injection (Fig. 5). Thus, the temporal distribution of [^{125}I]NGF-labelled neurons in L5 DRG shows clear evidence for consistent gradients in selective sizes of these cells.

As early as 6 h after injection, rows of labelling were found distributed along the longitudinal sections of the nerve fibres within the sensory branches of the spinal nerves, indicating individual fibres containing high amounts of radioactivity. At no time during the course of the experiment was labelling observed in the dorsal roots, ventral roots, or within the spinal cord. A comparison of the localization and staining intensity of labelled structures after injection of [^{125}I]NGF or HRP into crushed sciatic nerves at different survival times is shown in Table 1.

Retrograde transport of HRP in L5 DRG

HRP labelling in L5 DRG is observed in 57% of all neurons after 6 h, 75% after 8 h, 87% after 18 and 24 h, and 57% after 96 h of administration (Fig. 6A). The time course of HRP-labelling in L5 DRG (Fig. 6A) shows that there is a sharp increase followed by a slow decrease of labelling in DRG neurons. This indicates that the HRP which accumulates in the cell body must require considerable time to be either transported out of or broken down by the perikarya.

In contrast to the selective retrograde labelling of DRG neurons by [^{125}I]NGF, there was no clear gradient of HRP labelling in terms of neuronal size in L5 DRG. The labelling of small neurons by HRP overlapped the labelling of large neurons in the 9–96 h period. Fig. 7 demonstrates some examples of HRP-labelled neurons after 6 and 24 h. There is no clear preference in the sequence of labelling of either small or large neurons. The percentage of HRP-labelled neurons greater or smaller than 25 μm does not change significantly at all time points examined (Fig. 6B). Thus, 6 h after the injection, 40% of the labelled cells were small and 60% of labelled cells were large. At 24 h, the percentages were 45 and 55% for small and large cells, respectively. An examination of the size distribution of cells labelled with HRP (approximately 10 000 neurons from a total of three or four animals) after 6 and 24 h does not indicate a shift (Fig. 4b). These histograms completely overlap each other. The mean diameter of labelled neurons was 29 μm and 29 μm at 6 and 24 h, respectively. Clearly, both

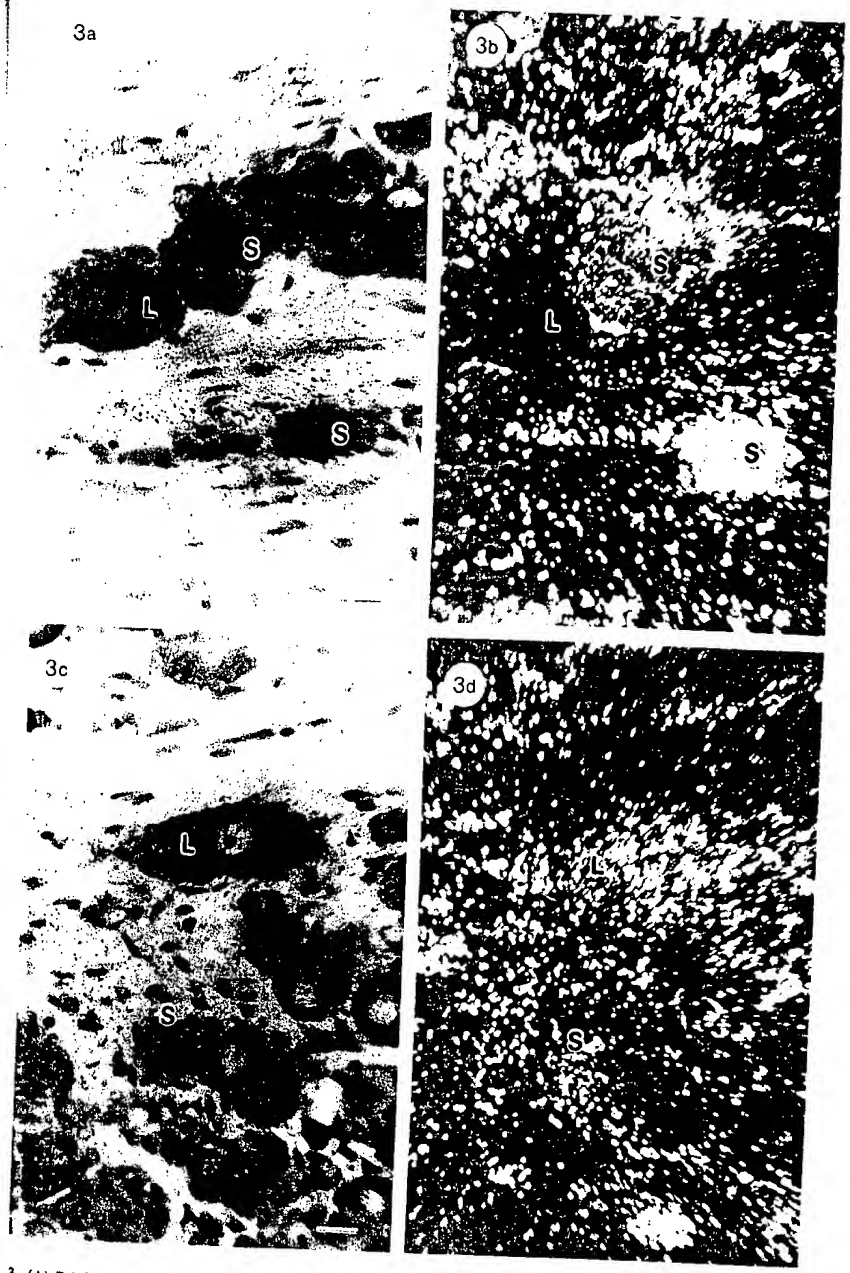


Fig. 3. (A) Brightfield autoradiograph of [^{125}I]NGF-labelled neurons 6 h after the injection which shows that small cells (indicated by S) are labelled and large cells (indicated by L) are not labelled. (B) Darkfield autoradiograph of the same section as in (A). (C) Brightfield autoradiograph of [^{125}I]NGF-labelled neurons 24 h after the injection which shows large cells also labelled. (D) Darkfield autoradiograph of the same section as in (C). Scale bars: 10 μm.

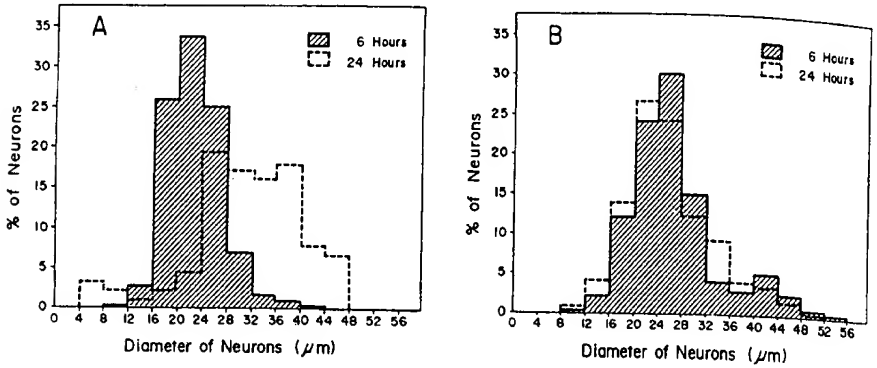


Fig. 4. (A) Histogram illustrating the size spectra of neuronal diameters in L5 DRG 6 h and 24 h after a [^{125}I]NGF injection into a crushed sciatic nerve. Note the histogram shifts to the right at 24 h which indicates an increase in the cell size of labelled neurons. (B) Histogram illustrating HRP injection into a crushed sciatic nerve. No shift in the histogram is observed in the HRP-injected ganglia.

Table 1. Comparison of differential labelling in L5 DRG with [^{125}I]NGF or HRP over time.

Survival time (h)	*Spinal nerve*	*Cell bodies DRG*	*Dorsal root*	*Ventral root*	*Axons or terminals in dorsal horn*	*Motor neuron cell bodies in ventral horn*
[^{125}I]NGF						
4	–	–	–	–	–	–
6	++	+	–	–	–	–
8	+++	+++	–	–	–	–
16	++	++	–	–	–	–
24	+	+	–	–	–	–
HRP						
4	–	–	–	–	–	–
6	+	+	–	–	–	–
8	++	++	–	–	–	–
16	+++	+++	+	+	+	++
24	+++	+++	++	++	+++	+++
96	+	++	+	+	++	+

– Indicates absence of labelling.
+ Indicates presence of labelling. Number of + represents the intensity of labelling: + means minimal but significant labelling; +++ means maximal labelling.

small and large neurons were equally well labelled after 6 and 24 h, respectively. Individual histograms of HRP-labelled neurons at each time point (6, 8, 16, 24 and 96 h) were remarkably similar to each other (data not shown), indicating that there was relatively little variability between time points. Further analysis of the relation of time of labelling to cell size shows that there is no change in the mean neuronal diameter over the period examined (Fig. 5).

At 4 h after the injection of HRP into the crushed sciatic nerve, no reaction product was observed in either dorsal and ventral roots or in any part of the corresponding spinal cord segments. At 8 h, labelled axons in the corresponding spinal nerves were seen. After 16 h, the intensity of the labelling was increased. Within the DRG, small neurons (10–25 μm) generally displayed a more intense labelling. However, some cells of this size were labelled rather

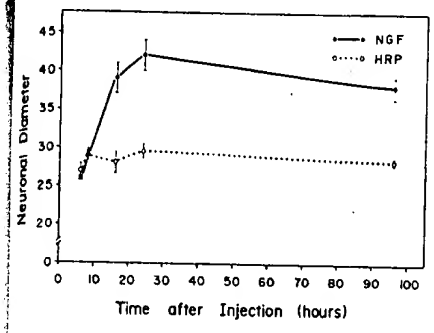


Fig. 5. The mean diameters of [^{125}I]NGF-labelled neurons and HRP-labelled neurons in L5 DRG at various times after the injection. Note the mean diameter of HRP-labelled neurons remains constant (30 μm) at all times after injection.

faintly, similar to larger neuronal cell bodies (25–45 μm). In addition to the increased labelling of DRG neuronal cell bodies and spinal nerve axons, a number of dorsal and ventral root axons showed traces of the label; labelling of the motor neurons was more distinct. The dorsal regions of the spinal cord did not show labelling until 16 h. At this time, labelling in Lissauer's tract and in the medial parts of the superficial laminae of the dorsal horn was evident. At 24 h after administration of HRP there was an intense labelling of DRG neurons and of their peripheral process, whereas a moderate staining was seen in axons of dorsal roots. Generally, the number of labelled fibres in the dorsal roots appeared definitely smaller than in the peripheral nerves and there was more reaction product in the peripheral than in the central process. At 24 h the majority of the dorsal and ventral root axons appeared labelled. Within the spinal cord, the number of labelled axons and terminals was increased in the first three laminae of the dorsal horn as well as in the tract of Lissauer. The motor neuron cell bodies and their dendrites and axons displayed a distinct labelling.

After a survival time of 96 h the number of labelled perikarya and axons in the DRG was significantly diminished; the intensity of the labelled structures was also decreased. Accordingly, fewer labelled fibres were observed in the dorsal roots and in spinal nerves. Labelling in the dorsal horn of the spinal cord was limited to the Lissauer's tract and the medial part of the dorsal horn. The intensity of labelling in motor neurons was also distinctly decreased. A summary of HRP-labelled structures at different survival times is shown in Table 1.

Discussion

The aim of the present study was, first, to determine whether the high selectivity of retrograde transport of NGF in sensory neurons is confined exclusively to a specific population of DRG neurons or is a property common to all neurons in the DRG. Second, we wished to compare the dynamics of retrograde transport and accumulation of NGF to HRP, a protein which has no biological effect but is, nevertheless, also retrogradely transported in sensory neurons.

Our results showed, in accordance with previous observations (Kristensson & Olsson, 1974; Yip &

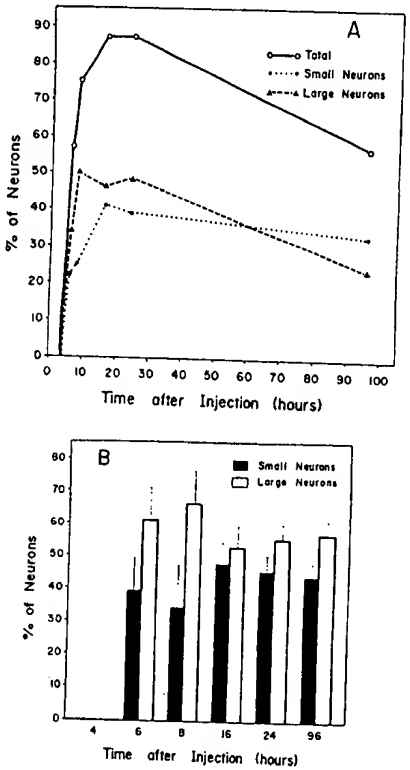


Fig. 6. Percentage of labelled neurons in L5 DRG at various times after injection of HRP into a crushed sciatic nerve. (A) Percentage of labelled cells in the total population of neurons in L5 DRG. (B) Comparison between the percentage of small (< 25 μm) labelled neurons and the percentage of large (> 25 μm) labelled neurons. There is no significant difference between the percentage of small labelled neurons and the percentage of large labelled neurons at all times observed.

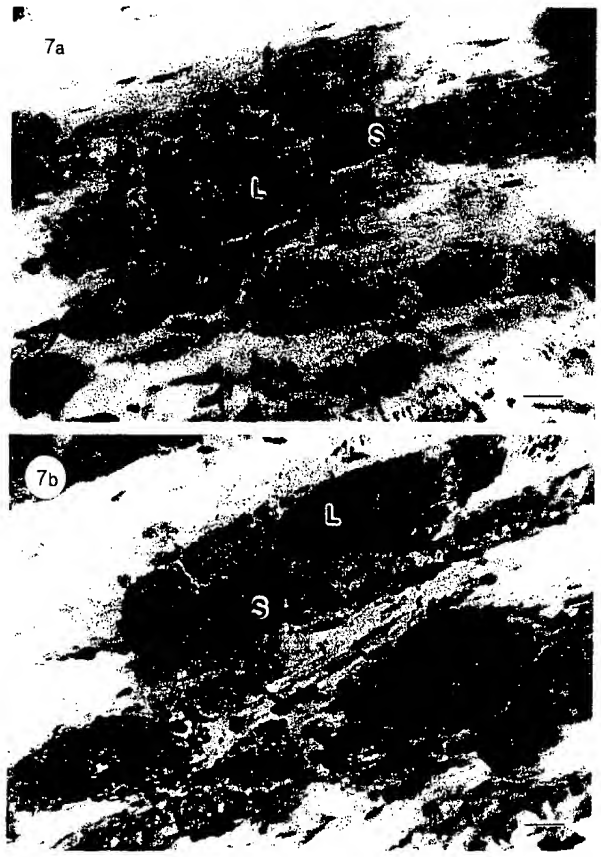


Fig. 7. Brightfield photomicrographs of HRP-positive labelled neurons at (A) 6 h and (B) 24 h after the injection. No observable difference in the labelling pattern of small (S) and large (L) neurons is demonstrated at either time point. Scale bars: 10 μm.

Johnson, 1983; Richardson & Riopelle, 1984), that application of either NGF or HRP to axotomized axons of peripheral nerves is followed by labelling of their perikarya. This suggests that much of the NGF or HRP is rapidly incorporated into organelles or bound to the receptors inside the crushed nerve. It is apparent from these experiments that the axon terminals are not essential for the uptake and retrograde transport of these macromolecules by peripheral nerves. This observation deserves some attention in the light of our previous finding which shows that uptake and retrograde transport of [^{125}I]NGF in axotomized sensory axons is specific and receptor-mediated (Yip & Johnson, 1983). It may, therefore, be suggested that NGF receptors are also present along the length of peripheral sensory axons or, alternatively, that NGF receptors are also retrogradely transported. In view of this observation and our recent observations (unpublished) which show that NGF receptors are retrogradely transported in sciatic nerves, the latter interpretation seems more likely. Previous experiments have shown that NGF is transported retrogradely and selectively accumulated in the population of large neurons in the DRG between 18 and 20 h after injection of [^{125}I]NGF into the forepaw (Stöckel *et al.*, 1975). Since it has been reported that retrograde transport of macromolecules can occur at several different rates (Kristensson *et al.*, 1971; Lubińska & Niemierko, 1971) and since it is known that the neurons of dorsal root ganglia are heterogeneous, it seemed possible that there is a difference in the labelling of neurons as a function of survival time. To test this hypothesis we studied the retrograde transport of [^{125}I]NGF in DRG neurons at various times after injection into crushed sciatic nerves. We found that the uptake and transport of NGF by DRG neurons (~ 4.5 cm from the crush site) proceeded generally in three phases that were dependent upon different survival times. (1) NGF was accumulated almost entirely (90%) in small neurons (25 μm or less) 6 and 8 h after the injection. (2) At 16 h after the injection of NGF into the crushed sciatic nerves, a more even distribution of large and small neurons contained NGF. (3) Subsequently, at 24 h after the injection, NGF was observed predominantly (83%) in large neurons (> 25 μm) and concomittantly there was a drastic decrease in the number of labelled small cells that were observed. The mean diameter of NGF-labelled neurons increased with longer survival time (from 22 μm at 6 h to 42 μm at 24 h). These results suggest that DRG neurons of different sizes transport or turnover [^{125}I]NGF at different rates. Ultimately, a similar percentage (~ 80%) of all cells in DRG at some time became labelled by both[^{125}I]NGF and HRP. This result is consistent with the observation that NGF deprivation during foetal development results in the death of the vast majority of DRG neurons and that the neurons are lost throughout the whole size spectrum (Johnson *et al.*, 1980). It remains to be seen whether the diversity in the NGF transport of different neurons is caused by varying transport capacities, by modified uptake rates of NGF at the crush site, by different turnover rates within the neuronal soma, or by some combination of these factors. There did not appear to be such a time-selective labelling of DRG with HRP. In our experiments, no clear trend of differential labelling of DRG neurons of different sizes by HRP was demonstrated. The mean diameter of HRP-labelled neurons (~ 30 μm) during the whole time course did not change. In general, small perikarya showed more intense labelling at an earlier stage than large perikarya. Similar observations have been demonstrated previously (Kristensson & Olsson, 1975; Grant *et al.*, 1979). This is probably because of a difference in the concentration of HRP accumulated in the small and large neurons. This finding, however, does not indicate that the small neurons are preferentially labelled at earlier survival times.

The concentration of tracer products observed in the DRG neuronal perikarya, at any of the time intervals, must reflect a balance of the rate of uptake of marker at the periphery, its rate of transport toward the cell body, its rate of degradation, and its possible anterograde transport back along the axons from the cell body. Therefore, the difference in labelling of neurons by different markers may be attributed to one or more of the factors mentioned. Labelling of perikarya of L5 DRG first appeared 6 h after injection of either [^{125}I]NGF or HRP to the sciatic nerve at the same distance (crushed at the tendon of *obturator internus*) to the ganglion. Thus, the fastest retrograde transport of [^{125}I]NGF and HRP occurred at a similar rate (~ 7.5 mm h^{-1}). However, the time span of the accumulated HRP in the neurons was much longer than that of [^{125}I]NGF. Transganglionic transport of HRP was observed in the dorsal horn. Similar findings have been reported in primary sensory neurons in rats (Grant *et al.*, 1979). There was no such labelling in the dorsal spinal cord after injection of [^{125}I]NGF. Since the apparent rate of degradation of the transported NGF seemed to be faster than HRP, this may contribute to the lack of transganglionic movement of NGF. Alternatively, the difference may result from a qualitative difference in the processing of ligands bound to the NGF receptors compared to agents transported by non-receptor-mediated processes.

In summary, this study has shown that different populations (in terms of sizes) of DRG neurons were retrogradely labelled by [^{125}I]NGF at different survival times. No such differences were seen in the labelling of neurons after injection of HRP. HRP also labelled motor neurons in the ventral horn and central terminals of DRG neurons by transganglionic transport. No transganglionic transport of [^{125}I]NGF was observed. The total number of DRG neurons which became labelled at some time with [^{125}I]NGF appeared to be the same as the number which became labelled by HRP, indicating that all DRG neurons retrogradely transport NGF. As a practical matter, in which retrograde labelling is used to define populations of neurons bearing NGF receptors, it is clear that observing retrogradely labelled DRG neurons at any given time after [^{125}I]NGF injection is not representative of the whole population of neurons capable of such transport. Whether this will be ultimately shown to be a consistent difference between specific 'receptor-mediated' transport and 'non-specific' transport is not known.

Acknowledgements

The authors thank Ms Patricia Lampe, Ms Patricia Osborne and Dr Arthur D. Loewy for their excellent assistance. We also thank Dr David McDougal for his helpful suggestions. This work was supported by NIH grants HL 20604 and NS 18071.

References

BOCCHINI, F & ANGELETTI, P. U. (1969) The nerve growth factor: purification as a 30,000 molecular weight protein. *Proceedings of National Academy of Sciences USA* **64**, 787–94.

CRAGG, B. G. (1970) What is the signal for chromatolysis? *Brain Research* **23**, 1–21.

GRANT, G., ARVIDSSON, J., ROBERTSON, B. & YGGE, J. (1979) Transganglionic transport of horseradish peroxidase in primary sensory neurons. *Neuroscience Letters* **12**, 23–8.

HAMBURGER, V. (1975) Cell death in the development of the lateral motor column of the chick embryo. *Journal of Comparative Neurology* **160**, 535–46.

HARMAR, A. J. & KEEN, P. (1981) Peptide biosynthesis in sensory ganglia. In *Neuropeptides: Basic and Clinical Aspects* (edited by FINK, G. & WHALLEY, L. J.), pp. 259–71. Edinburgh: Churchill Livingstone.

HENDRY, I. A. (1975) The retrograde axonal transport of nerve growth factor. *Brain Research* **94**, 87–97.

HENDRY, I. A. & IVERSEN, L. L. (1973) Changes in tissue and plasma concentration of nerve growth factor following removal of submaxillary glands in adult mice and the effects on the sympathetic nervous system. *Nature* **243**, 500–4.

JOHNSON, E. M., GORIN, P. D., BRANDIES, L. D. & PEARSON, J. (1980) Dorsal root ganglion neurons are destroyed by exposure *in utero* to maternal antibody to nerve growth factor. *Science* **210**, 916–18.

KALINA, M. & WOLMAN, M. (1970) Correlative histochemical and morphological study on the maturation of sensory ganglion cells in the rat. *Histochemie* **22**, 100–8.

KESSLER, J. A. & BLACK, I. B. (1980) Nerve growth factor stimulates the development of substance P in sensory ganglia. *Proceedings of the National Academy of Sciences USA* **77**, 649–52.

KORNBLUM, H. I. & JOHNSON, E. M., Jr (1982) Time and dose dependence of effects of nerve growth factor on sympathetic and sensory neurons in neonatal rats. *Brain Research* **234**, 41–51.

KRISTENSSON, K. (1975) Retrograde axonal transport of protein tracers. In *The Use of Axonal Transport for Studies of Neuronal Connectivity* (edited by COWAN, W. M. & CUÉNOD, M.), pp. 69–82. Amsterdam: Elsevier.

KRISTENSSON, K. & OLSSON, Y. (1974) Retrograde transport of horseradish peroxidase in transected axons. I. Time relationships between transport and induction of chromatolysis. *Brain Research* **79**, 101–10.

KRISTENSSON, K. & OLSSON, Y. (1975) Retrograde transport of horseradish peroxidase in transected axon. II. Relationship between rate of transfer from the site of injury to the perikaryon and onset of chromatolysis. *Journal of Neurocytology* **4**, 653–61.

KRISTENSSON, K., OLSSON, Y. & SJÖSTRAUD, J. (1971) Axonal uptake and retrograde transport of exogenous proteins in the hypoglossal nerve. *Brain Research* **32**, 399–406.

LaVAIL, J. H. (1975) The retrograde transport method. *Federation Proceedings* **34**, 1618–24.

LAWSON, S. N. (1979) The postnatal development of large light and small dark neurons in the mouse dorsal root ganglia: a statistical analysis of cell number and sign. *Journal of Neurocytology* **8**, 275–94.

LAWSON, S. N. & NICKELS, S. M. (1980) The use of morphometric techniques to analyze the effect of neonatal capsaicin treatment on rat dorsal root ganglia and dorsal roots. *Journal of Physiology* **303**, 12P.

LIEBERMAN, A. R. (1976) Sensory ganglia. In *The Peripheral Nerve* (edited by LANDON, D. N.), pp. 188–278, London: Chapman and Hall.

LUBÍNSKA, L. & NIEMIERKO, S. (1971) Velocity and intensity of bidirectional migration of AChE in transected nerves. *Brain Research* **27**, 329–42.

MARCHALONIS, J. J. (1969) An enzyme method for the trace iodination of immunoglobulins and other proteins. *Biochemical Journal* **113**, 229–305.

MESULAM, M. M. (1978) Tetramethylbenzidine for horseradish peroxidase neurochemistry. A non-carcinogenic blue reaction product with superior sensitivity for visualizing neural afferent and efferents. *Journal of Histochemistry and Cytochemistry* **26**, 106–17.

NAUTA, H. J. W., PRITZ, M. B. & LASEK, R. J. (1974) Afferents to the cat caudoputamen studied with horseradish peroxidase. An evaluation of a retrograde neuroanatomical research method. *Brain Research* **67**, 219–38.

PARFIANOWICZ, J., HAWRYLKO, S., PIETRZAK, J. & KMIEC, B. (1971) Morphology and cytochemistry of the nerve cells of the spinal ganglia. *Folia Morphologica* (Warsaw) **30**, 423–31.

RICHARDSON, P. M. & RIOPELLE, R. J. (1984) Uptake of nerve growth factor along peripheral and spinal axons of primary sensory neurons. *Journal of Neuroscience* **4**, 1683–9.

STÖCKEL, K., PARAVICINI, U. & THOENEN, H. (1974) Specificity of the retrograde axonal transport of nerve growth factor. *Brain Research* **76**, 413–21.

STÖCKEL, K., SCHWAB, M. E. & THOENEN, H. (1975) Specificity of retrograde transport of nerve growth factor (NGF) in sensory neurons: a biochemical and morphological study. *Brain Research* **89**, 1–14.

THOENEN, H. & BARDE, Y.-A. (1980) Physiology of nerve growth factor. *Physiological Review* **60**, 1285–335.

YIP, H. K. & JOHNSON, E. M., Jr (1983) Retrograde transport of nerve growth factor in lesioned goldfish retinal ganglion cells. *Journal of Neuroscience* **3**, 2172–82.

YIP, H. K., RICH, K. M., LAMPE, P. A. & JOHNSON, E. M., Jr (1984) The effects of nerve growth factor and its antiserum on the postnatal development and survival after injury of sensory neurons in rat dorsal root ganglia. *Journal of Neuroscience* **4**, 2986–92.

Journal of Neurocytology **15**, 799–815 (1986)

Distribution of the adhesion molecules N-CAM and L1 on peripheral neurons and glia in adult rats

RHONA MIRSKY[1], KRISTJÁN R. JESSEN[1], MELITTA SCHACHNER[2] and CHRISTO GORIDIS[3]

[1]*Department of Anatomy and Embryology, University College London, Gower Street, London WC1E 6BT, UK*
[2]*Department of Neurobiology, University of Heidelberg, Im Neuenheimer Feld 504, 6900, Heidelberg, FRG*
[3]*Centre d'Immunologie, INSERM–CNRS de Marseille-Luminy, 13288 Marseille Cedex 9, France*

Received 29 April 1986; revised 2 June 1986; accepted 9 June 1986

Summary

There is considerable evidence that the cell surface glycoproteins N-CAM and L1 are important mediators of cell–cell adhesion in the nervous system, at least during development. Numerous studies have been devoted to the molecular properties of these proteins and their adhesion role in embryonic and early postnatal development. Much less is known about their importance in mature tissues. A rigorous and comprehensive description of the cell distribution of these molecules in the adult nervous system would clearly form a useful baseline for functional and biochemical studies. In the present work we have addressed this issue and studied the distribution of N-CAM and L1 throughout adult, as opposed to developing, rat peripheral nervous tissue. Particular attention was paid to the ganglia of the enteric nervous system, since adhesion mechanisms within these ganglia are likely to be placed under unusual demands.

We report, for the first time, the presence of N-CAM and L1 on mature sensory, sympathetic and enteric neurons in adult rats. Thus, immunostaining of cell suspensions or short-term cultures showed N-CAM and L1 surface labelling on sympathetic and both large and small dorsal root sensory neurons. Both antigens were also present on the surface of enteric neurons in cultures prepared from 10-day-old rats and neonatal guinea pigs. Immunostaining of sections of enteric ganglia from adults indicated that both molecules were also expressed by mature enteric neurons. In sections of mature sciatic nerve neither N-CAM nor L1 immunoreactivity were detected at the site where the plasma membrane of myelinated axons meets the ad-axonal plasma membrane of the myelin-forming Schwann cell. Thus, both N-CAM and L1 were detected on all major classes of peripheral neurons, while their levels in the plasma membrane of myelinated axons may be significantly down-regulated.

Similarly, both N-CAM and L1 were present on all major classes of non-myelin-forming peripheral glia in adult rats. This includes the enteric glial cells of the myenteric ganglia, non-myelin-forming Schwann cells in the sciatic nerve, sympathetic trunk and fine autonomic nerves in the gut wall, and the satellite glial cells of sympathetic and dorsal root sensory ganglia. In contrast, myelin-forming Schwann cells did not express detectable levels of N-CAM and only very low levels of L1, which was mainly located near the nodes of Ranvier.

On the basis of these findings the prediction would be that, with the exception of myelinated fibres, N-CAM and L1 operate in parallel to link neurons and glia throughout the adult rat PNS. It remains to be seen whether myenteric ganglia, a part of the nervous system exposed to an unusual degree of mechanical stress, possess additional adhesive mechanisms. Myelin-forming Schwann cells appear to differ from other peripheral glia in their adhesive interactions with neuronal membranes since they do not express detectable levels of N-CAM and and show only low levels of L1.

Introduction

The evidence that the membrane glycoproteins N-CAM and L1 play a crucial role in the mechanical adhesion between neural cells derives from many biochemical, immunochemical and immunohistochemical studies, the majority of which have focused on embryonic or early postnatal tissue (e.g. Goridis *et al.*, 1983; Lindner *et al.*, 1983; Edelman, 1984; Rutishauser, 1984). Much less is known about their functional importance in the adult nervous system. Interestingly, recent studies suggest that the N-CAM present in mature tissues is more adhesive than the N-CAM present in early development, indicating that these adhesion molecules may be at least as important in adult animals (Edelman, 1985; Rutishauser *et al.*, 1985). A rigorous and comprehensive description of the localization of these molecules on different cell types in the adult nervous system would clearly form a useful baseline for further biochemical and functional studies. In this paper we have addressed this issue by studying the distribution of N-CAM and L1

0300-4864/86 $03.00 + .12

nation of the fetal biological clock *in utero*. Science *220:* 969–971.

Rusak, B., and I. Zucker (1979) Neural regulation of circadian rhythms. Physiol. Rev. *59:* 449–526.

Schwartz, W. J., and H. Gainer (1977) Suprachiasmatic nucleus: Use of ^{14}C-labeled deoxyglucose uptake as a functional marker. Science *197:* 1089–1091.

Schwartz, W. J., L. C. Davidson, and C. B. Smith (1980) *In vivo* metabolic activity of a putative circadian oscillator, the rat suprachiasmatic nucleus. J. Comp. Neurol. *189:* 157–167.

Sharp, F. R., T. S. Kilduff, S. Bzorgchami, H. C. Heller, and A. F. Ryan (1983) The relationship of local cerebral glucose utilization to optical density ratios. Brain Res. *263:* 97–104.

Sokoloff, L., M. Reivich, C. Kennedy, M. H. Des Rosiers, C. S. Patlak, K. D. Pettigrew, O. Sakurada, and M. Shinohara (1977) The ^{14}C deoxyglucose method for the measurement of local cerebral glucose utilization: Theory, procedure, and normal values in the conscious and anesthetized albino rat. J. Neurochem. *28:* 897–916.

Tannenbaum, G., and J. B. Martin (1976) Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. Endocrinology *98:* 540–548.

0270-6474/84/0407-1683$02.00/0

The Journal of Neuroscience
Vol. 4, No. 7, pp. 1683–1689
July 1984

UPTAKE OF NERVE GROWTH FACTOR ALONG PERIPHERAL AND SPINAL AXONS OF PRIMARY SENSORY NEURONS[1]

P. M. RICHARDSON[2] AND R. J. RIOPELLE

Division of Neurosurgery, McGill University, Montreal, Canada, and Division of Neurology, Queen's University, Kingston, Canada

Received August 22, 1983; Revised January 13, 1984; Accepted January 13, 1984

Abstract

To investigate the distribution of nerve growth factor (NGF) receptors on peripheral and central axons, [^{125}I]NGF was injected into the sciatic nerve or spinal cord of adult rats. Accumulation of [^{125}I]NGF in lumbar dorsal root ganglia was monitored by gamma emission counting and radioautography.

[^{125}I]NGF, injected endoneurially in small quantities, was taken into sensory axons by a saturable process and was transported retrogradely to their cell bodies at a maximal rate of 2.5 to 7.5 mm/hr. Because very little [^{125}I]NGF reached peripheral terminals, the results were interpreted to indicate that receptors for NGF are present on nonterminal segments of sensory axons. The specificity and high affinity of NGF uptake were illustrated by observations that negligible amounts of gamma activity accumulated in lumbar dorsal root ganglia after comparable intraneural injection of [^{125}I] cytochrome C or [^{125}I]oxidized NGF.

Similar techniques were used to demonstrate avid internalization and retrograde transport of [^{125}I]NGF by intraspinal axons arising from dorsal root ganglia. Following injection of [^{125}I]NGF into lumbar or cervical regions of the spinal cord, neuronal perikarya were clearly labeled in radioautographs of lumbar dorsal root ganglia.

Sites for NGF uptake on primary sensory neurons in the adult rat are not restricted to peripheral axon terminals but are extensively distributed along both peripheral and central axons. Receptors on axons provide a mechanism whereby NGF supplied by glia could influence neuronal maintenance or axonal regeneration.

Nerve growth factor (NGF) is necessary for the development and function of sensory neurons (Gorin and Johnson, 1979, 1980; Kessler and Black, 1980; Goedert et al., 1981; Schwartz et al., 1982; Otten and Lorez, 1983); NGF, or a similar molecule, is released by Schwann cells or other cells that are present within peripheral nerve trunks (Burnham et al., 1972; Riopelle et al., 1981; Richardson and Ebendal, 1982). If NGF-like activity of endoneurial origin is physiologically important (Varon and Bunge, 1978), receptors for NGF are to be expected in axonal membrane along the course of peripheral sensory fibers. In previous studies of axonal internalization and retrograde transport of [^{125}I]NGF *in vivo* (Hendry et al., 1974; Stoeckel et al., 1975; Johnson et al., 1978; Dumas et al., 1979), uptake has been demonstrated at or near peripheral sympathetic and sensory terminals, but the possible existence of receptors on more proximal axonal segments has not been examined systematically. *In vitro*, [^{125}I]NGF has been shown by radioautography to bind to cell bodies, neurites, and neurite terminals of cultured sympathetic and dorsal root ganglion neurons (Kim et al., 1979; Carbonetto and Stach, 1982; Rohrer and Barde, 1982). From observations of [^{125}I]NGF uptake and transport in adult rats, we conclude that specific receptors for NGF are found on peripheral sensory axons within the sciatic nerve and also on spinal axons of primary sensory neurons.

Materials and Methods

Preparation of [^{125}I]NGF. NGF was prepared from submandibular glands of male Swiss-Webster mice (Jackson Laboratories) according to the method of Mobley et al. (1976) with substitution of a continuous gradient of NaCl concentration for the last elution (Chapman et al., 1981). In bioassay with dissociated chick

[1] This work was supported by the Medical Research Council of Canada and the Multiple Sclerosis Society of Canada. We wish to thank Valerie Issa, Monica Altares, and Shizuye Faulkner for technical assistance and Evelyn Domond for secretarial assistance.

[2] To whom correspondence should be addressed, at Division of Neurosurgery, Montreal General Hospital, 1650 Cedar Avenue, Montreal, Canada, H3G 1A4.

sensory neurons (Riopelle and Cameron, 1981), maximal neurite outgrowth was obtained at approximately 70 pg/ml of NGF. For radioiodination (Sutter et al., 1979), a mixture of NaI (5 μCi, Amersham Searle), NGF (5 μg), hydrogen peroxide (5 μl of a 0.003% solution at 0 and 30 min), and phosphate buffer (0.1 M, pH 7.4 to a total volume of 50 μl) were incubated for 1 hr at room temperature. Fifty microliters of cold 0.4% acetic acid and 200 to 400 μl of acetate buffer (0.5 M, pH 4.0) with bovine serum albumin (1 mg/ml) and protamine sulfate (1 mg/ml) were then added, and the mixture was dialyzed for 36 to 48 hr against two or three changes of 0.05 M acetate buffer (pH 4.0). The efficiency of labeling was 50 to 65%, and after dialysis >95% of gamma activity was precipitable with 10% trichloroacetic acid. Six preparations of [^{125}I]NGF from two batches of NGF were used with final concentrations of 10 to 38 ng/μl and specific activities of 34 to 84 cpm/pg (approximately 30 to 80 μCi/μg). For dose-response curves, [^{125}I]NGF was diluted with saline or mixed with unlabeled NGF, and final concentrations were verified with gamma counting. Cytochrome C (Sigma Chemical Co.) and NGF with two oxidized tryptophan residues (Cohen et al., 1980) were also radioiodinated by the lactoperoxidase technique. The final concentrations and specific activities were 12 ng/μl and 18 cpm/pg for [^{125}I]cytochrome C and 11 ng/μl and 14 cpm/pg for [^{125}I]oxidized NGF. Iodinated proteins were injected within 2 weeks of preparation.

Injections. Female Sprague-Dawley rats weighing 150 to 200 gm (Charles River Breeding Labs) were anesthetized with pentobarbital, and the sciatic nerve was exposed in the thigh. Through a glass micropipette connected to a Hamilton syringe and filled with mineral oil (Beitz and King, 1976), 1 μl of a given solution was injected into the sciatic nerve near the origin of the nerve to biceps femoris. In control experiments, injection was, if necessary, further distal, and the sciatic nerve was crushed with jeweller's forceps either at the site of injection or 1.0 to 2.0 cm proximally. In two instances, the tibial, peroneal, sural, and biceps branches of the sciatic nerve were all cut at the time of injection to prevent [^{125}I]NGF from reaching sensory axon terminals.

For intraspinal injections, laminectomies were performed with microsurgical technique at high cervical or high lumbar levels. After subdural injection of paraffin oil to prevent extravasation in the CSF, [^{125}I]NGF (or another ^{125}I-protein) was injected in two to four sites to a total volume of 2 to 4 μl.

Counting of gamma activity. Four to 24 hr after injection of [^{125}I]NGF, rats were anesthetized and perfused with 4% formaldehyde, 0.5% glutaraldehyde in phosphate buffer. The fourth and fifth lumbar dorsal root ganglia (L4 and L5 DRG), identified by reference to the sacrum, were removed, and their gamma emission was counted in an LKB-Wallac 1270 Rackgamma counter.

SDS-polyacrylamide gel electrophoresis. Twelve L4 or L5 DRG, collected 10 hr after intraneural administration of [^{125}I]NGF, were homogenized in an aqueous solution of 1% SDS and 2% β-mercaptoethanol. The homogenate was concentrated to 0.3 ml by evaporation, heated to 100°C for 5 min, and centrifuged. Fifty-microliter aliquots of the supernatant were electrophoresed with a discontinuous buffer system (Laemmli, 1970) and resolving gel containing 12% polyacrylamide and 0.1% SDS. Each lane of the gel was cut into 10-mm slices which were counted individually.

Radioautography. Selected DRG were fixed, osmicated, dehydrated, and embedded in plastic. Sections, 1 μm thick, were dipped in Kodak NTB-2 emulsion, stored in lightproof boxes for 1 month, developed with Kodak D-19 solution, stained with toluidine blue, and mounted in glycerine.

Results

One hour after injection of [^{125}I]NGF (1 μl, 8 ng) into the sciatic nerve, 97% of the activity within the nerve was contained in a segment 1 cm long; at 12 hr, 96% of the intraneural activity (35% of the injected activity) was recovered in a 3.5 cm segment of nerve (Fig. 1). These data delineate limits for diffusion of [^{125}I]NGF within the nerve.

Eight or more hours after injection of [^{125}I]NGF into the sciatic nerve, significant gamma activity was consistently detected in ipsilateral L4 and L5 DRG (Fig. 2*a*). Small and variable amounts of gamma activity were also counted in L3 and L6 ganglia, but none was found in the lumbar spinal cord. Neither crushing the nerve at the site of injection nor isolating the nerve from its peripheral connections by sectioning distal branches changed the accumulation of activity in L4 and L5 DRG. Nerve crush 1.5 to 2.0 cm proximal to the injection site completely prevented gamma activity from reaching lumbar DRG (Table I); crush 1.0 cm proximal blocked 90% of gamma activity from accumulating in L4 and L5 DRG. After injection of comparable amounts of [^{125}I]cytochrome C or [^{125}I]oxidized NGF, gamma activity in lumbar DRG was not significantly higher than background (Table I). These control experiments exclude diffusion along the nerve, hematogenous spread, and nonspecific uptake as spurious causes of labeling in DRG of experimental rats.

To determine the rate of transport of [^{125}I]NGF in the sciatic nerve, rats were sacrificed 4, 8, 12, 18, and 24 hr after intraneural injection in the thigh (Fig. 2*b*). No activity reached L4 and L5 DRG in 4 hr; counts were maximal at 12 to 18 hr and fell by 24 hr. Given that the injection site was 3.5 cm from the 14 ganglion and that [^{125}I]NGF diffused 0.5 to 1.5 cm proximally, the rate of transport was estimated at 2.5 to 7.5 mm/hr for the fastest particles.

In another group of rats, a dose-response curve was obtained by injecting different quantities (0.1 to 74 ng in 1 μl) of [^{125}I]NGF into the sciatic nerve and counting activity in L4 and L5 DRG after 11 hr (Fig. 2*c*). Double reciprocal analysis of data (inverse of accumulation versus inverse of injection) indicated a saturable process with maximal accumulation per DRG of 29 pg (1.1 fmol) and half-maximal uptake at 4 ng (0.2 pmol) (Fig. 2*d*). The true maximal accumulation is, perhaps, slightly higher because accumulation was 38 pg in one series of rats injected with freshly dialyzed [^{125}I]NGF. The amounts of NGF injected were insufficient to study probable additional uptake at lower affinity (Dumas et al., 1979).

1.

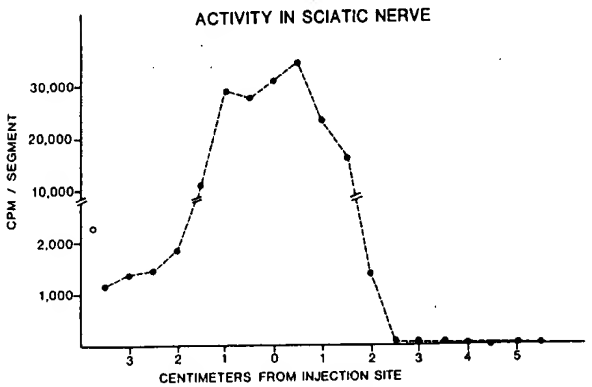


Figure 1. Activity in segments, 5 mm long, of the sciatic nerve 12 hr after injection of [^{125}I]NGF (1 μl, 8 ng, 500,000 cpm) into nerve in upper thigh. ●, mean value per segment in two injected nerves; ○, mean total activity in L4 and L5 DRG. Thirty-seven percent of injected activity was recovered in the nerve and two DRG at 12 hr. Very high counts near the site of injection indicate the extent of diffusion; negligible counts in distal segments show lack of anterograde axonal transport; moderate counts in proximal segments and DRG are compatible with retrograde axonal transport.

After SDS-polyacrylamide gel electrophoresis, 77% of the recovered gamma activity migrated in the penultimate slice before the front as did a standard molecule of molecular weight = 14,400 (α-lactalbumin). This information is compatible with previous evidence that NGF is transported retrogradely in an intact form (Hendry et al., 1974; Stoeckel et al., 1975; Johnson et al., 1978; Dumas et al., 1979).

In radioautographs of L4 and L5 DRG, label was concentrated in neurons rather than non-neuronal cells or the extracellular space. Neurons of all sizes were labeled and, in some ganglia, more than a third of neurons contained 10 or more grains.

Uptake from lumbar spinal cord. Very little [^{125}I]NGF diffused into L4 and L5 spinal roots after lumbar intraspinal injection. In one such rat, 10 hr after injection, the total activity in a 1 cm segment of the spinal cord at the site of injection was 218,000 cpm, and the average activities in consecutive 5-mm segments of the L4 and L5 roots were 1721, 543, 240, 255, and 386 cpm. Thus, injection of [^{125}I]NGF into the lumbar spinal cord did not result in a large pool of activity in the lumbar spinal roots.

After intraspinal injection, as after intraneural injection, [^{125}I]NGF was consistently recovered from L4 and L5 DRG (Fig. 3*a*). In radioautographs, activity was concentrated in neurons. Accumulation was prevented if dorsal roots were crushed or avulsed at the time of injection (Table I). Twelve hours after injection of [^{125}I]cytochrome C, the mean activity in L4 and L5 DRG averaged 8 cpm above background.

The time course of accumulation of [^{125}I]NGF in lumbar DRG after injection into the lumbar spinal cord (Fig. 3*b*) showed insignificant activity at 4 hr, peak counts at 12 hr, and some loss from the ganglia by 24 hr. As the L4 and L5 dorsal roots are 2.5 to 3.0 cm long, these data are compatible with maximal retrograde axonal transport at 3.1 to 7.5 mm/hr.

A dose-response curve (Fig. 3*c*) and double reciprocal plotting of data (Fig. 3*d*) again suggested saturable uptake with maximal accumulation of 14 pg (0.5 fmol)/DRG and half-maximal uptake with 14 ng (0.5 pmol).

Uptake from high spinal levels. In a total of 11 rats (Table II), [^{125}I]NGF from four preparations was injected into the dorsal columns and dorsal column nuclei at the craniocervical junction. Subsequently, mean accumulation per lumbar DRG was 0.5 to 1.3 pg in the four groups. In radioautographs of DRG in one well labeled animal, approximately 6% of neurons were labeled (Fig. 4). Counts in lumbar DRG after cervical injection of [^{125}I]cytochrome C did not exceed background.

Following injection of [^{125}I]NGF into the midthoracic spinal cord (32 ng in 3 μl), the accumulation per lumbar DRG (0.8 ± 0.1 pg) was similar to that after cervical injection of the same batch of [^{125}I]NGF. The uptake was unchanged by deliberate injury to the spinal cord immediately rostral to the site of injection.

Discussion

NGF uptake by peripheral axons. NGF receptors are deduced to be present along the course of normal sensory axons in the sciatic nerve because the intraneuronal accumulation of [^{125}I]NGF injected into the endoneurium cannot be entirely explained by diffusion to peripheral

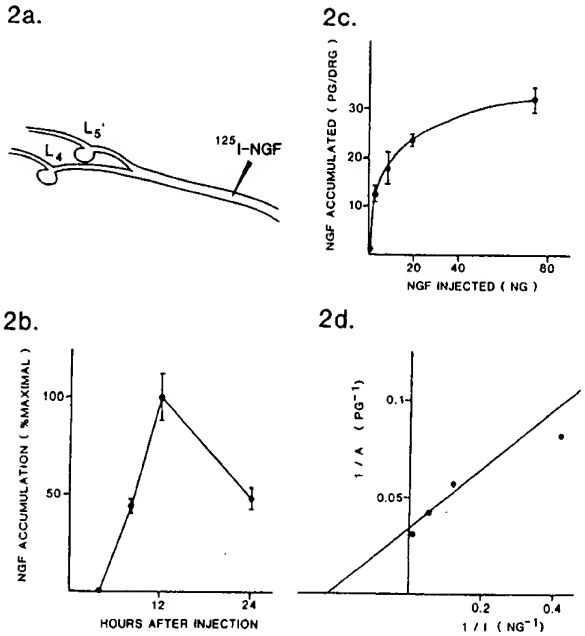


Figure 2. *a*, Diagram illustrating experiments in which [^{125}I]NGF was injected into the sciatic nerve in the upper thigh and gamma activity was subsequently counted in L4 and L5 DRG. *b*, Accumulation of NGF in L4 or L5 DRG of rats sacrificed 0 to 24 hr after injection of [^{125}I]NGF (1 μl, 11 ng) into sciatic nerve in upper thigh. The mean ± SEM for 12 ganglia (6 nerves) is expressed as a percentage of the accumulation at 12 hr. *c*, Accumulation of NGF in L4 or L5 DRG of rats sacrificed 11 hr after injection of 0.3 to 74 ng of [^{125}I]NGF (1 μl) into sciatic nerve in upper thigh. A mixture of labeled and unlabeled NGF was used for the highest concentration; serial dilutions of [^{125}I]NGF were used for all other points. Data are the mean ± SEM (picograms per DRG) for 12 to 16 ganglia and 6 to 8 nerves. *d*, Double reciprocal plot of data in *c*. The reciprocal of the *y* intercept = the maximal amount of [^{125}I]NGF accumulated per DRG by high affinity system = 29 pg (1.1 fmol). The reciprocal of the *x* intercept = the amount of injected [^{125}I]NGF yielding half-maximal saturation = 4 ng (0.2 pmol).

TABLE I

Comparison of mean accumulation per DRG in one experimental group and three control groups after intraneural injection, and in one experimental group and two control groups after intraspinal injection

Activity in L4 and L5 DRG was counted 12 to 24 hr after injection

	Injection into Sciatic Nerve		Injection into Lumbar Spinal Cord	
	Injection	Accumulation	Injection	Accumulation
	cpm	*cpm/DRG*	*cpm*	*cpm/DRG*
[^{125}I]NGF	500,000	1071	700,000	409
[^{125}I]NGF with nerve or root crush	500,000	3	1,800,000	6
[^{125}I]Cytochrome C	400,000	5	800,000	8
[^{125}I]Oxidized NGF	300,000	3		

receptors or by injury of axons at the time of injection. Indeed, it was originally reported (Hendry et al., 1974), although not subsequently emphasized, that intact terminals are not necessary for NGF internalization by sympathetic axons. NGF receptors were specific in their recognition of injected molecules and in their distribution among types of axons. [^{125}I]Cytochrome C and [^{125}I] oxidized NGF were not taken up at high affinity as was [^{125}I]NGF; somatic motor axons did not internalize and transport [^{125}I]NGF as did sensory axons (and, presumably, sympathetic axons). Although [^{125}I]NGF accumulated in large and small neurons in DRG, it is not known whether axonal membrane on all dorsal root ganglion neurons is equally responsive to NGF. The maximal rate of retrograde axonal transport of NGF in sensory axons was found to be slightly slower than previously calculated (Stoeckel et al., 1975) but similar to that for NGF in sympathetic axons (Hendry et al., 1974) and for other proteins in general (Grafstein and Forman, 1980).

Quantitative considerations. The amounts of NGF accumulating in sensory ganglia after intraneural injection and in sympathetic ganglia after intraocular injection are similar, as are the amounts of injected NGF necessary

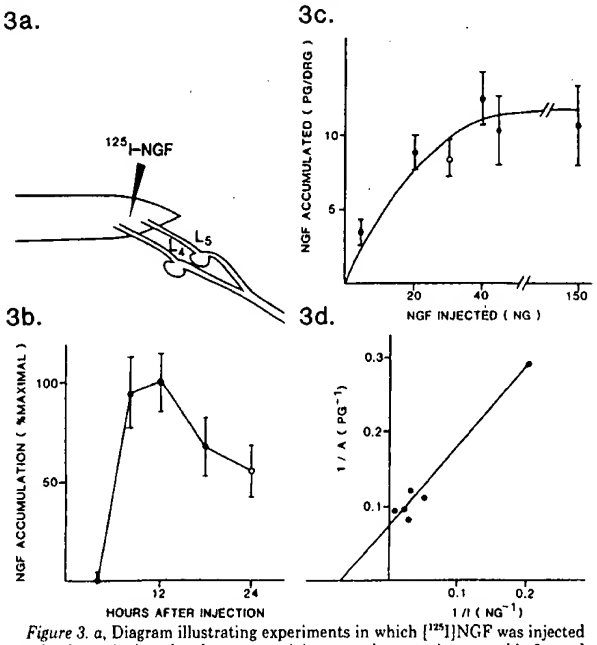


Figure 3. *a*, Diagram illustrating experiments in which [^{125}I]NGF was injected into lumbar spinal cord and gamma activity was subsequently counted in L4 and L5 DRG. *b*, Accumulation of NGF in L4 or L5 DRG in rats sacrificed 0 to 24 hr after injection of [^{125}I]NGF into the lumbar spinal cord. Two preparations of [^{125}I]NGF were injected: O, 40 ng in 4 μl; ●, 100 ng in 3 μl. The mean ± SEM for 16 ganglia (4 rats) is expressed as a percentage of the accumulation at 12 hr. *c*, Accumulation of NGF in L4 or L5 DRG in rats sacrificed 12 hr after injection of [^{125}I]NGF (4 μl) into the lumbar spinal cord. O, Three different batches of [^{125}I] NGF were used. Data are the mean ± SEM (picograms of [^{125}I]NGF per DRG) for 16 ganglia (4 rats). A mixture of labeled and unlabeled NGF was used for the highest concentration; serial dilutions of [^{125}I]NGF were used for all other points. *d*, Double reciprocal plot of data in *c*. The reciprocal of the *y* intercept = the maximal amount of [^{125}I]NGF accumulated per DRG by high affinity system = 14 pg (0.5 fmol). The reciprocal of the *x* intercept = the amount of injected [^{125}I] NGF yielding half-maximal saturation = 14 ng (0.5 pmol).

TABLE II

Cervical injection of [^{125}I]NGF

Results of four experiments (11 rats) in which [^{125}I]NGF was injected into the dorsal columns and dorsal column nuclei at the bulbospinal junction. Gamma activity in L4 and L5 was consistently above background levels.

Injection		Hours to Sacrifice	Accumulation
ng	μl		*pg/DRG ± SEM*
20	2	18	0.6 ± 0.1
32	3	24	0.6 ± 0.1
42	2	24	0.8 ± 0.1
53	2	24	1.3 ± 0.2

for half-maximal uptake (Johnson et al., 1978). The maximal accumulation and half-saturation point of the dose-response curve are assumed to reflect the density and affinity of axonal receptors for NGF (Dumas et al., 1979). The density of receptors in the sciatic nerve can be very approximately estimated at 10,000/mm length of each sensory axon if it is assumed that [^{125}I]NGF reaching the L4 or L5 DRG is taken up by 6000 axons over a 10-mm segment of nerve, that each receptor internalizes one molecule of NGF only, and that recycling of receptors is negligible during a few hours. The dissociation equilibrium constant (K_d) of axonal receptors cannot be calculated precisely from these data because the concentration of [^{125}I]NGF at the axonal membrane after endoneurial injection is unknown. It remains to be proved that NGF receptors with K_d in the order of 10^{-11} M are present on adult rat axons as on embryonic chick neurons (Sutter et al., 1979; Riopelle et al., 1980).

The finding of NGF receptors along the course of peripheral sensory axons suggests that NGF released by endoneurial cells might have local paracrine effects. However, the amount of injected [^{125}I]NGF yielding half-saturable uptake in sensory axons (4 ng) is two orders of

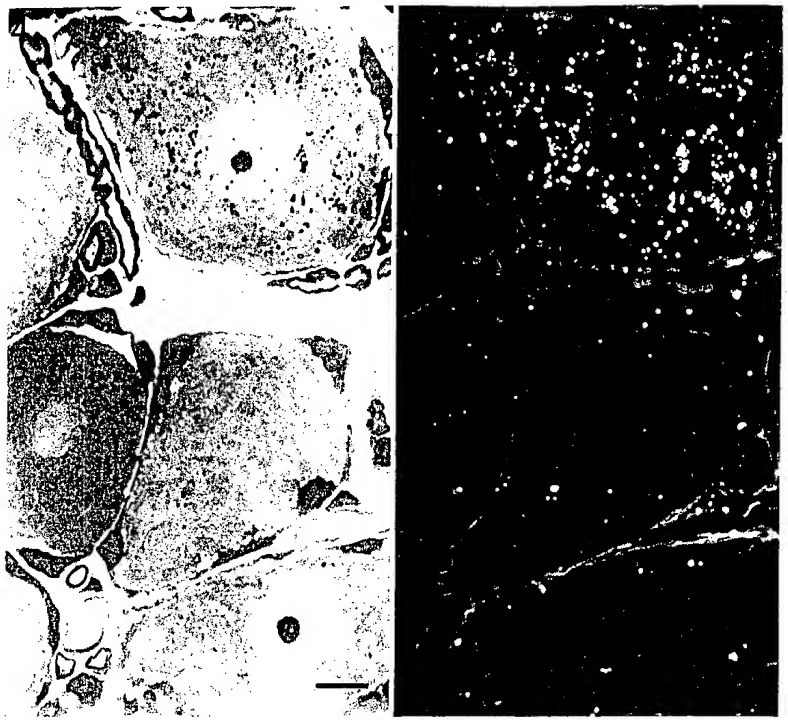


Figure 4. Light- and darkfield radioautographs of L5 DRG in a rat sacrificed 24 hr after injection of [^{125}I]NGF (2 μl, 53 ng, 4.5 × 10^6 cpm) into dorsal column nuclei and high cervical dorsal columns. Labeling in one neuron is well above background level (*scale bar* = 10 μm).

magnitude greater than the estimated content of NGF-like activity per centimeter length of normal rat sciatic nerve (Ebendal and Richardson, 1983). Quantitative analysis of endogenous ligand-receptor interactions awaits knowledge of the distribution of NGF-like activity within the endoneurium and the deployment of NGF receptors on axonal membrane of nodal and internodal segments.

NGF uptake by spinal axons. Previous evidence that receptors for NGF are present within the central nervous system (Frazier et al., 1974; Schwab et al., 1979) is corroborated by the finding of labeled neurons in radioautographs of lumbar DRG after cervical injection of [^{125}I]NGF (Fig. 4). In other words, ensheathment by Schwann cells is not a necessary condition for the manifestation of NGF receptors. The apparent difference in affinities of NGF uptake from the lumbar spinal cord and the sciatic nerve is probably due to a larger volume of diffusion in the spinal cord rather than to different dissociation equilibrium constants for spinal and peripheral receptors. Sites for NGF internalization could be on terminal or nonterminal regions of spinal axons, and their density per unit area of axon membrane in the spinal cord could be equal or less to that in peripheral nerves. The function of NGF receptors in the maintenance and regeneration (Richardson et al., 1982) of sensory axons in the spinal cord is unknown.

References

Beitz, A. J., and G. W. King (1976) An improved technique for the microinjection of horseradish peroxidase. Brain Res. *108:* 175–179.

Burnham, P., C. Raiborn, and S. Varon (1972) Replacement of nerve-growth factor by ganglionic non-neuronal cells for the survival *in vitro* of dissociated ganglionic neurons. Proc. Natl. Acad. Sci. U. S. A. *69:* 3556–3560.

Carbonetto, S., and R. W. Stach (1982) Localization of nerve growth factor bound to neurons growing nerve fibers in culture. Dev. Brain Res. *3:* 463–473.

Chapman, C. A., B. E. C. Banks, C. A. Vernon, and J. M. Walker (1981) The isolation and characterisation of nerve growth factor from the prostate gland of the guinea-pig. Eur. J. Biochem. *115:* 347–351.

Cohen, P., A. Sutter, G. Landreth, A. Zimmermann, and E. M. Shooter (1980) Oxidation of tryptophan-21 alters the biological activity and receptor binding characteristics of mouse nerve growth factor. J. Biol. Chem. *255:* 2949–2954.

Dumas, M., M. E. Schwab, and H. Thoenen (1979) Retrograde axonal transport of specific macromolecules as a tool for characterizing nerve terminal membranes. J. Neurobiol. *10:* 179–197.

Ebendal, T., and P. M. Richardson (1983) Bioassay and radioimmunoassay of NGF-like activity in rat peripheral nerve. Soc. Neurosci. Abstr. *9:* 839.

Frazier, W. A., L. F. Boyd, M. W. Pulliam, A. Szutowicz, and R. A. Bradshaw (1974) Properties and specificity of binding sites for ^{125}I-nerve growth factor in embryonic heart and brain. J. Biol. Chem. *249:* 5918–5923.

Goedert, M., K. Stoeckel, and U. Otten (1981) Biological importance of the retrograde axonal transport of nerve growth factor in sensory neurons. Proc. Natl. Acad. Sci. U. S. A. *78:* 5895–5898.

Gorin, P. D., and E. M. Johnson (1979) Experimental autoimmune model of nerve growth factor deprivation: Effects on developing peripheral sympathetic and sensory neurons. Proc. Natl. Acad. Sci. U. S. A. *76:* 5382–5386.

Gorin, P. D., and E. M. Johnson, Jr. (1980) Effects of long-term nerve growth factor deprivation on the nervous system of the adult rat: An experimental autoimmune approach. Brain Res. *198:* 27–42.

Grafstein, B., and D. S. Forman (1980) Intracellular transport in neurons. Physiol. Rev. *60:* 1167–1283.

Hendry, I. A., K. Stoeckel, H. Thoenen, and L. L. Iversen (1974) The retrograde axonal transport of nerve growth factor. Brain Res. *68:* 103–121.

Johnson, E. M., Jr., R. Y. Andres, and R. A. Bradshaw (1978) Characterization of the retrograde transport of nerve growth factor (NGF) using high specific activity ^{125}I-NGF. Brain Res. *150:* 319–331.

Kessler, J. A., and I. B. Black (1980) Nerve growth factor stimulates the development of substance P in sensory ganglia. Proc. Natl. Acad. Sci. U. S. A. *77:* 649–652.

Kim, S. U., R. Hogue-Angeletti, and N. K. Gonatas (1979) Localization of nerve growth factor receptors in sympathetic neurons cultured *in vitro.* Brain Res. *168:* 602–608.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227:* 680–685.

Mobley, W. C., A. Schenker, and E. M. Shooter (1976) Characterization and isolation of proteolytically modified nerve growth factor. Biochemistry *15:* 5543–5551.

Otten, U., and H. P. Lorez (1983) Nerve growth factor increases substance P, cholecystokinin and vasoactive intestinal polypeptide immunoreactivities in primary sensory neurones of newborn rats. Neurosci. Lett. *34:* 153–158.

Richardson, P. M., and T. Ebendal (1982) Nerve growth activities in rat peripheral nerve. Brain Res. *246:* 57–64.

Richardson, P. M., U. M. McGuinness, and A. J. Aguayo (1982) Peripheral nerve autografts to the rat spinal cord: Studies with axonal tracing methods. Brain Res. *237:* 147–162.

Riopelle, R. J., and D. A. Cameron (1981) Neurite growth promoting factors of embryonic chick—ontogeny, regional distribution and characteristics. J. Neurobiol. *12:* 175–186.

Riopelle, R. J., M. Klearman, and A. Sutter (1980) Nerve growth factor receptors: Analysis of the interaction of βNGF with membranes of chick embryo dorsal root ganglia. Brain Res *199:* 63–77.

Riopelle, R. J., R. J. Boegman, and D. A. Cameron (1981) Peripheral nerve contains heterogeneous growth factors that support sensory neurons in vitro. Neurosci. Lett. *25:* 311–316.

Rohrer, H., and Y. -A. Barde (1982) Presence and disappearance of nerve growth factor receptors on sensory neurons in culture. Dev. Biol. *89:* 309–315.

Schwab, M. E., U. Otten, Y. Agid, and H. Thoenen (1979) Nerve growth factor (NGF) in the rat CNS: Absence of specific retrograde axonal transport and tyrosine hydroxylase induction in locus coeruleus and substantia nigra. Brain Res. *168:* 473–483.

Schwartz, J. P., J. Pearson, and E. M. Johnson (1982) Effect of exposure to anti-NGF on sensory neurons of adult rats and guinea pigs. Brain Res. *244:* 378–381.

Stoeckel, K., M. Schwab, and H. Thoenen (1975) Specificity of retrograde transport of nerve growth factor (NGF) in sensory neurons: A biochemical and morphological study. Brain Res. *89:* 1–14.

Sutter, A., R. J. Riopelle, R. M. Harris-Warrick, and E. M. Shooter (1979) Nerve growth factor receptors: Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. J. Biol. Chem. *254:* 5972–5982.

Varon, S. S., and R. P. Bunge (1978) Trophic mechanisms in the peripheral nervous system. Annu. Rev. Neurosci. *1:* 327–361.